Conception de nitrones amphiphiles aux propriétés de piégeage et antioxydantes supérieure

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THESE

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SPECIALITE: CHIMIE ORGANIQUE

Design of amphiphilic nitrones with improved spin-trapping and antioxidant properties

Par

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Soutenance prévue le 16 Décembre 2014 devant le Jury composé de :

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AVANT-PROPOS

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Des publications :

- Rosselin, M.; Choteau, F.; Zéamari, K.; Nash, K. M.; Das, A.; Lauricella, R.; Lojou, E.; Tuccio, B.; Villamena, F. A. and Durand, G.; Reactivities of Substituted α-Phenyl-*N*-*tert*-butyl Nitrones. *The Journal of Organic Chemistry*, **2014**, *79*, 6615.

- Rosselin, M.; Tuccio, B.; Villamena, F. A. and Durand, G.; Electrochemical and Spin-Trapping Properties of *para*-substituted α-Phenyl-*N*-*tert*-butyl Nitrones, *New Journal of Chemistry*, **in preparation**.

- Rosselin, M.; Zéamari, K.; Choteau, F.; Hericher, Q.; Tuccio, B. and Durand, G.; α-Phenyl-*N*-cyclohexyl Nitrones: Preparation and Use as Spin-Traps, **in preparation**.

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Des communications orales en Anglais :

- Design of amphiphilic nitrones with improved spin-trapping and antioxidant properties Rosselin, M.; Tuccio, B.; Villamena, F.A. and Durand, G.; 1st Balard Chemistry Conferences, Self Assembly of Biomolecules, La Grande Motte, 12-15 Octobre 2014.

- Synthesis of α-Phenyl-*N*-*tert*-butylnitrone derivatives with improved antioxidant and spin-trapping properties. Rosselin, M.; Tuccio, B.; Villamena, F.A. and Durand, G.; 8th Organic chemistry meeting (RCOM8), Marseille, 12-13 Juin 2014.

- Design of α-Phenyl-*N*-*tert*-butylnitrone derivatives with improved antioxidant properties. Rosselin, M.; Tuccio, B.; Villamena, F.A. and Durand, G.; 21st Young research meeting in therapeutic chemistry, Montpellier, 24-25 Mars 2014.

Des communications par affiche :

- Rosselin, M. ; Tuccio, B. ; Villamena, F.A. and Durand, G. ; Design of amphiphilic nitrones with improved spin-trapping and antioxidant properties; Self assembly of biomolecules; La Grande Motte; 12-15 Octobre 2014.

- Rosselin, M; Choteau, F.; Zéamari, K.; Nash, K.; Das, A.; Lauricella, R.; Lojou, E.; Tuccio, B.; Villamena, F.A. and Durand, G.; Spin-trapping & antioxidant properties of α-Phenyl-*N*-*tert*-butyl Nitrone derivatives; French association of Electron Paramagnetic Resonance; Lille, 2-5 Juin 2014.

- Rosselin, M; Choteau, F.; Tuccio, B.; Lojou, E.; Villamena, F.A. and Durand, G.; Reactivity of beta-substituted α-Phenyl-*N*-*tert*-butyl Nitrones; 1st Young research's mediterranean workshop; Montpellier; 17-18 Octobre 2013.

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LIST OF ABBREVIATIONS

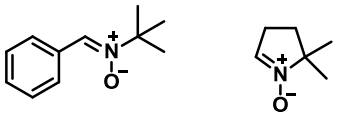
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
Ac_2O	anhydride acetic
AcOH	acetic acid
AZN	azulenyl nitrone
BAEC	bovine aortic endothelial cells
BBB	blood brain barrier
Boc	*tert*-butoxycarbonyl
CDI	1,1'-carbonyldiimidazole
CH	cyclohexane
CH_2Cl_2	dichloromethane
CH_3CN	acetonitrile
CMC	critical micelle concentration
DCC	N,N'-Dicyclohexylcarbodiimide
DEA	diethylamine
DLS	dynamic light scattering
DMF	dimethylformamide
DMAP	4-dimethylaminopyridine
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
DMSO	dimethylsufoxide
EDG	electron-donating group
EPR	electron paramagnetic resonance
EtOAc	ethyl acetate
EtOH	ethanol
EWG	electron-withdrawing group
Fmoc	fluorenylmethyloxy carbonyl
H_2	hydrogen
hfcc's	hyperfine coupling constants

H_2O_2	hydrogen peroxide
HNE	4-hydroxynonenal
HOBt	hydroxybenzotriazole
HOMO	highest occupied molecular orbital
HPLC	high pressure liquid chromatography
LOX	lipoxygenase
LPO	lipid peroxidation
LUMO	lowest unoccupied molecular orbital
MTT	3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium-bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
$ONOO^-$	peroxinitrite
OtBu	*tert*-butyl ester
PBN	α-phenyl-*N*-*tert*-butylnitrone
$Pb(OAc)_4$	lead tetracetate
$Pd_{/c}$	palladium-on-carbon catalyst
pH	hydrogen potential
Pyr	pyridin
ROS	reactive oxygen species
SIN-1	3-morpholinosynonimine hydrochloride
STAZN	stilbazulenyl nitrones
TBAP	tetrabutyl ammonium perchlorate
TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TES	triethylsilane
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Z	carboxybenzyl

INTRODUCTION

Avant l'apparition du vivant, l'oxygène était quasiment absent de l'atmosphère. Il n'y a finalement que 2,5 milliards d'années qu'il est produit en excès et qu'il est devenu un composant de l'atmosphère terrestre, pour atteindre la proportion de l'ordre de 20% que nous connaissons aujourd'hui. Progressivement et à l'exception de certain organismes anaérobies, l'oxygène est devenu indispensable à la vie des animaux, plantes et bactéries en leur permettant de produire de l'énergie. Cette production d'énergie est caractérisée par la formation régulière de composés prooxydants souvent délétères, que l'on appelle Espèces Oxygénées et Azotées Réactives (EOR/EAR). Les cellules se sont adaptées à cet environnement en se dotant d'un système de défense enzymatique capable de procéder à la détoxification de ces métabolites. Néanmoins, les EOR/EAR ne sont pas seulement nocives vis-à-vis de l'organisme, elles exercent également des fonctions indispensables. Malheureusement, lorsque ces espèces réactives sont produites en quantité trop importante et que la cellule ne parvient plus à réguler cette formation, le compartiment cellulaire connaît alors un état appelé « stress oxydant ». Dans plusieurs maladies graves, notamment celles liées au vieillissement, le stress oxydant est impliqué dans l'étiologie de la maladie. C'est le cas par exemple de cancers, de pathologies oculaires et de maladies neurodégénératives. Par conséquent, depuis de nombreuses années les chercheurs tentent de développer différentes thérapies afin de limiter les effets néfastes de ce phénomène. L'une des stratégies consiste à renforcer le système de défense cellulaire par l'administration d'antioxydants naturels ou synthétiques. Malheureusement, la prise d'antioxydants naturels n'apporte pas toujours les bénéfices escomptés et ces composés peuvent même être prooxydants dans certains cas. Il a donc fallu se tourner vers l'élaboration de molécules antioxydantes synthétiques, qui sont très souvent des analogues structuraux d'antioxydants naturels. Parmi les analogues synthétiques largement utilisés, on peut citer le Trolox qui est un dérivé hydrosoluble de la vitamine E.

Une autre voie explorée a consisté à utiliser dans le domaine biologique, des molécules dont le rôle initial était de mettre en évidence et de caractériser les radicaux libres sur le plan physico-chimique. En effet, l'identification d'espèces radicalaires par Résonance Paramagnétique Electronique (RPE) a permis d'obtenir de précieuses informations sur la nature et la structure des radicaux. Cette technique très sensible s'appuie sur le piégeage de spin permettant, par addition d'un composé diamagnétique au milieu radicalaire, de former des adduits de spin beaucoup plus stables que l'on peut donc étudier facilement. Cette technique a permis d'améliorer la compréhension de processus pathologiques liés au stress oxydant et a vu l'émergence de nouvelles molécules capables de piéger les radicaux libres d'origine biologiques, c'est le cas des nitrones. Il existe deux grandes catégories de nitrones : les nitrones linéaires dérivés de l'α-Phényl-*N*-*tert*-Butyl-Nitrone (PBN) et les nitrones cycliques dérivés de 5,5-DiMéthyl-1-Pyrroline-*N*-Oxide (DMPO).



PBN **DMPO**

De nombreuses études ont été réalisées sur les effets protecteurs des nitrones et la PBN s'est avérée plus efficace que la DMPO en tant qu'agent à propriétés thérapeutiques. Son caractère partiellement amphiphile, qui lui permet d'être à la fois soluble en milieux aqueux et en milieux lipidiques, serait à l'origine de sa bonne biodisponibilité *in vivo*. Plusieurs équipes de chercheurs ont tenté d'améliorer l'efficacité thérapeutique de ces antioxydants synthétiques en développant différents analogues ou en favorisant leur ciblage vers des sites endommagés par la production d'espèces oxydantes.

C'est dans cette optique que depuis de nombreuses années, notre laboratoire développe différent type de structures monomoléculaires amphiphiles capables de transporter un principe actif dans l'organisme par modulation de sa balance hydrophile-lipophile. L'objectif de ces

structures est de mieux contrôler le passage transmembranaire d'un principe actif et ainsi lui assurer un meilleur ciblage. Cette stratégie a donc été appliquée aux nitrones. Les résultats ont montré que le greffage de la PBN ou de la DMPO sur une structure amphiphile augmente significativement leur biodisponibilité et leur activité biologique. Ce principe a aussi été appliqué à d'autres antioxydants et différents types de transporteurs amphiphiles ont été synthétisés, dans le but d'améliorer l'activité biologique du principe actif étudié.

Les travaux menés au cours de ma thèse ont consisté à poursuivre cette thématique de recherche en modulant l'activité de la PBN. Le premier chapitre de ce manuscrit est présenté sous forme d'introduction bibliographique, faisant l'état de l'art des différentes structures de nitrones connues à ce jour. La PBN étant reconnue depuis plusieurs années pour ses activités protectrices, plusieurs auteurs ont synthétisé des analogues afin d'améliorer ses propriétés et sa biodisponibilité. Leurs travaux ce sont d'abord focalisés sur des modifications chimiques assez simples portant sur la partie *N*-terminale et aromatique de la PBN, puis les études se sont orientées vers la synthèse de nitrones aromatique dérivées de la PBN jusqu'au développement de dérivés au ciblage plus spécifique. Dans ce chapitre d'introduction bibliographique, l'accent sera mis à la fois sur la capacité des nitrones à piéger les radicaux libres ainsi que sur leurs propriétés physico-chimiques et leur activité biologique.

Dans le second chapitre, nous nous sommes intéressés à différentes modulations autour de la PBN afin d'améliorer ses propriétés intrinsèques. Ce chapitre est découpé en trois parties. Dans un premier temps, nous avons effectué des modifications sur la partie *N*-*tert*-butyl de la PBN en greffant un, deux ou trois substituant(s) afin d'augmenter la réactivité de la fonction nitrone. Ainsi, nous avons étudié l'influence des différents substituants sur les propriétés physico-chimiques et biologiques de nos composés. La seconde partie de notre travail s'est portée sur l'étude de composés modifiés en para du groupement phényl, préalablement synthétisés au laboratoire. Dans cette partie nous nous sommes focalisés sur l'influence des

effets électroniques générés par les substituants sur les propriétés physico-chimiques. Enfin, dans la dernière partie de ce chapitre, nous avons modifié le groupement *tert*-butyl par un groupement bifonctionnalisé composé d'un cyclohexane et d'une chaîne alkyle substituable. D'une part, le cyclohexane présent en α du groupe nitronyl devrait permettre d'augmenter la rigidité globale de la molécule, stabiliser le radical formé et ainsi améliorer ses capacités de piégeage d'EOR/EAR. D'autre part, la fonction acide carboxylique présente sur la chaîne alkyle pourra être greffée à un autre composé, permettant ainsi un ciblage spécifique de la nitrone. Après exploration de ces trois types de modifications autour de la PBN, l'objectif à plus long terme est de combiner les structures les plus intéressantes afin de synthétiser un dérivé, substitué à la fois en *para* du phényl et en *N*-*tert*-butyl de la nitrone, présentant des propriétés physico-chimiques optimales.

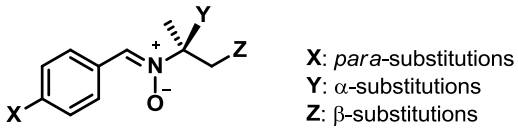


Figure 1. Structure générale de la PBN substituée.

Dans le troisième chapitre de ce manuscrit, nous nous sommes penchés sur le ciblage spécifique de nitrones dans l'organisme. La première partie de ce travail a consisté à greffer le motif PBN sur des structures amphiphiles afin de favoriser le passage transmembranaire. Nous avons ainsi poursuivit les travaux du laboratoire sur la synthèse de transporteurs amphiphiles à fixation latérale comportant un acide aminé jouant le rôle de bras espaceur entre la tête hydrophile, la partie hydrophobe et l'antioxydant. La nouveauté dans cette partie a été de développer des transporteurs comportant deux acides aminés consécutifs afin de greffer deux antioxydants sur un même transporteur amphiphile (structures schématisées ci-dessous). Nous avons d'abord synthétisé un transporteur amphiphile portant deux motifs nitrones, dans le but d'étudier l'effet de divalence. Ensuite, nous avons synthétisé un

transporteur amphiphile permettant de coupler deux antioxydants différents, par exemple une nitrone et un autre antioxydant, afin d'envisager un effet de synergie.

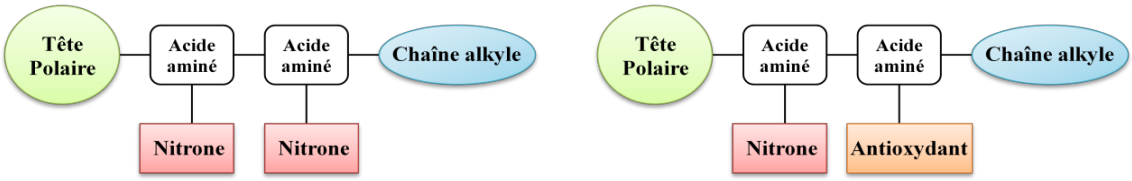


Figure 2. Représentation schématique des deux transporteurs amphiphiles.

Enfin, dans la deuxième et dernière partie de ce chapitre, nous avons voulu apporter un ciblage actif à nos nitrones et cibler cellules responsables du mélanome, les mélanocytes. En collaboration avec l'université de Clermont-Ferrand, nous avons modifié la partie hydrophile du transporteur amphiphile par un groupement benzamide capable de cibler spécifiquement les mélanocytes. Les dérivés benzamides ont une certaine affinité pour la mélanine, synthétisée dans les mélanocytes, qui a elle-même montré un effet protecteur dans la dégénérescence oculaire liée à l'âge. Ce type de pathologie étant également associé à une production excessive d'EOR, nous avons trouvé intéressant de combiner la PBN à un groupement benzamide afin de cibler spécifiquement les mélanocytes. Deux dérivés ont été synthétisés et caractérisés, leur activité biologique sur un modèle rétinien est actuellement en cours d'étude.

Nous avons fait le choix de rédiger ce manuscrit en Anglais, sous forme de Thèse d'articles. Ainsi, les parties expérimentales sont décrites à la fin de chaque sous-partie. Afin d'éviter les redondances nous avons consigné les références bibliographiques à la fin de chaque chapitre. Les différentes techniques physico-chimiques utilisées au cours de cette thèse sont présentées en annexe de ce manuscrit.

Chapter I

Nitrone derivatives as Spin-Traps and Therapeutics: From chemical modification to specific-targeting

Part of this work is currently being drafted as a review article.

Reactive oxygen species (ROS) that are mostly free radicals along with reactive nitrogen species (RNS) are known to play a dual role in biological systems, since they can be either harmful or beneficial to the living organisms. ROS and RNS at low concentration have been demonstrated to regulate a variety of normal functions, such as signal transduction, mitogenetic response and defence against infectious agents. ROS/RNS are balanced with an endogenous antioxidant system, which includes enzymatic and non-enzymatic defences. There is a second class of antioxidant that is obtained from the diet constituting the so-called exogenous antioxidant system. However, excessive production or deficient degradation of ROS/RNS in cells leads to an *in vivo* redox unbalance which can be deleterious. This is commonly termed as *oxidative stress*. Indeed ROS/RNS are responsible of severe damages to biological macromolecules such as proteins, DNA and lipids. Therefore, this biological state is often associated to several pathologies and syndromes such as ischemia-reperfusion, chronic inflammation, ageing as well as some genetic diseases to name a few.[1, 2] To prevent oxidative damages, therapeutic strategies using natural antioxidants have been extremely developed and promising results have been reported. Unfortunately, clinical trials have provided inconstant results[3-6] and therefore other antioxidant strategies have been developed. Synthetic antioxidants have been developed with two main goals: having more potent agents than the natural ones in preventing oxidative stress, and/or having agents that exhibit a specific targeting properties so as on site protection is achieved.

Spin-trapping properties. Nitrones were initially designed as spin traps for detecting transient free radicals using electron paramagnetic resonance (for a recent review see Villamena & Zweier, 2004).[7] As shown in Figure 1.1, free radicals react with the diamagnetic nitrone to from a paramagnetic spin adduct whose half-life is significantly longer than that of the parent radical. Among the family of nitrone, two classes have been mainly

developed, the cyclic ones derived from the 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and the linear ones derived from the α-phenyl-*N*-*tert*-butyl nitrone (PBN) (Figure 1.2).

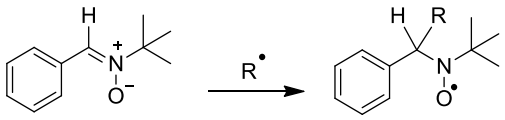


Figure 1.1. Mechanism of spin trapping by linear PBN.

Cyclic nitrones have been widely employed as probes for the detection of free radicals because of their better ability to trap oxygen-centered radicals compared to linear ones. Therefore, several analogues of DMPO have been designed over the past two decades. One can cite the phospshorylated analogue 5-diethoxyphosphoryl-5-methyl-1pyrroline *N*-oxide (DEPMPO)[8] as well as the ester analogs, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO)[9] and 2-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO).[10] Recently, a new amido derivative AMPO was reported to have the highest rate constant of superoxide trapping, followed by EMPO, both DEPMPO and DMPO, having the slowest reactivity.[11, 12]

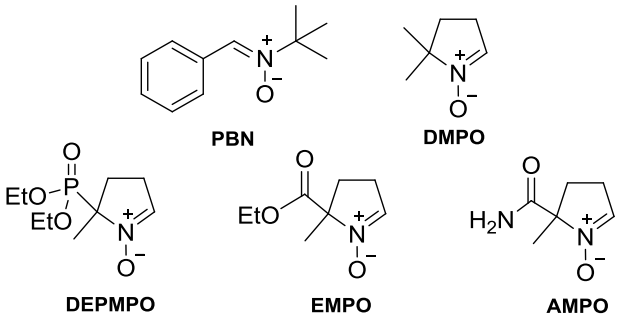


Figure 1.2. Chemical structure of linear PBN, cyclic DMPO and some derivatives.

Although linear nitrones are generally considered to be poorer traps compared to cyclic ones, they have been also employed in spin trapping experiments to trap carbon-centered radicals either in organic or aqueous phase. Toxicity of nitrone compounds is low rendering their use in biological systems possible without inducing any severe side effect.[13-15] However, the

use of both the linear PBN series and the cyclic DMPO one as spin-traps in biological milieu has encountered some limitation. Indeed, the DMPO-superoxide spin adducts decomposes quickly in polar environment and its biological uses to extracellular spin trapping are limited by its hydrophilicity.[16-18] On the other hand, because of its lipophilicity, the distribution of PBN within tissues and cells is much higher than that of the hydrophilic DMPO[16-18] but its spin-trapping properties are rather limited with low spin-adduct stability.

Synthesis of nitrones. Several methods are available for the synthesis of nitrones.[19, 20] The condensation between aldehydes and *N*-monosubstituted hydroxylamine is the most widely used method for the preparation of nitrones. One-pot synthesis is also used through *in situ* reduction of a nitro group to its hydroxylamine, which is subsequently condensed with a benzaldehyde derivative. Another method consists in the *N*-oxidation of an imine intermediate directly into nitrone using strong oxidants such as peracids.[21, 22] Finally, *N*-alkylation of aldoximes also leads to nitrone formation.[23, 24]

Biological properties. Mode of action of nitrones. Since the seminal work of Novelli *et al*.[25] nitrone spin traps have been widely used as antioxidants in several biological models and their use as pharmaceutical agents have been extensively reviewed.[26, 27] The biological activity of nitrones had been first explained by their radical trapping activity, however, experimental evidence have suggested other mechanisms. For instance, the concentrations used for spin trapping experiments in chemical media are roughly one thousand times higher than those commonly employed in biological studies of protection (10-50 μM). In addition, concentration of nitrones in target tissues is usually inferior to 50 μM, which is not sufficient to quench all the radical species. These findings support the invalidation of the spin-trapping mechanism as the primary mode of action of nitrones. In addition, there are strong evidence that PBN act to quell signal transduction processes providing therefore, potent anti-inflammatory and anti-apoptotic properties.[26, 28] Nitrones

also exhibit NO-releasing properties which may be partly responsible for their pharmacological properties.[29, 30] Hensley *et al*. demonstrated that PBN interacts with the mitochondrial complex I by inhibiting complex-I stimulated H_2O_2 flux.[31]

Considering the very broad activity of nitrones, there has been extensive research on the development of novel nitrone-based compounds with improved biological and spin trapping properties as well as enhanced intra-cellular compartmentalization. The chemical and pharmacological properties of nitrones depend mainly on the connectivity as well as on the nature and the position of the substituents on the nitrone group. Several analogs of PBN, and to a lesser extend of DMPO, have been synthesized during the last years combining in their structures chemical functions that can improve the intrinsic properties of the spin-trap compound. Chemical modifications have been made either on the phenyl ring and/or on the *N-tert*-butyl group of PBN so as to improve the reactivity of the nitronyl group and the stability of the nitroxide spin-adduct. Modifications have also been applied on the nitronyl group such as substitutions on the carbon atom of the nitrone function with a heretoaryl or an azulenyl group. Moreover, to target nitrones to specific tissues and/or cellular compartments, the nitronyl group was grafted onto specific cargo such as lipophilic cations, peptides or amphiphilic carriers. The different strategies used to improve the potency of PBN-Based nitrones either as spin-traps or as therapeutics will be described in this chapter.

1. Modifications on the phenyl ring and the *N-tert*-butyl groups

β-phosphorylated derivatives. Oxygen-centered radical trapping of nitrones is of particular interest as theses radicals are involved in many chemical and biological process. Among the most widely employed spin-traps, PBN is much more lipophilic than DMPO, making PBN more suitable for biological applications. However, the use of PBN against superoxide-hydroperoxyl and hydroxyl radicals has shown limitation. Therefore, various PBN analogs

bearing substituents on the phenyl ring were developed, mainly by Janzen and co-workers.[32-35] However, these compounds presented similar limitations that are rapid disappearance of hydroxyl and superoxide radicals in aqueous solution, and very similar EPR spectra with close hyperfine splitting constants (hfsc) leading to possible misinterpretation. A phosphorylated derivative of DMPO, denoted DEPMPO and represented in Figure 1.3, showed interesting spin-trapping properties with half-lives values about fifteen times higher than that of DMPO in aqueous solutions.[36-38] This improvement of the hydroperoxyl spin-adduct stability was found to be induced by the presence of the diethylphosphoryl group which tends to stabilize the aminoxyl obtained during the trapping. Therefore, the use of a phosphoryl group was also extended to linear PBN and 4-PyOBN nitrones. Structures of the corresponding *N*-benzylidene-1-diethoxyphosphoryl-1-methylamine *N*-oxide (PPN) and 1-diethoxyphosphoryl-1-methylamine *N*-oxide (4-PyOPN) are represented in Figure 1.3.[39] Replacement of the methyl group by a diethylphosphoryl group led to a significant increase of the spin-adduct half-lives.

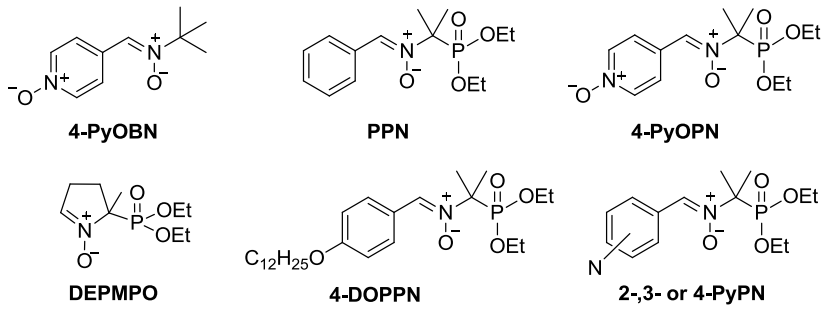


Figure 1.3. Structure of 4-PyOBN and β-phosphorylated nitrones.

Several PPN-type nitrones, by varying substitutions on the aryl group, were synthesized (Figure 1.4).[40] Except 4-DOPPN, all PPN-type nitrones were able to trap carbon-centered radicals and formed intense and persistent spin adducts in aqueous and organic media. PPN and 4-PyOPN were the most potent of this series in trapping superoxide radical in aqueous environment, with acceptable decay kinetics as well. One of the advantages of the PPN-type

series is that their various spin-adducts are easily identified by their EPR spectra, because of the existence of a strong hyperfine coupling with the phosphorus nucleus.

Ester derivatives. Tuccio and co-workers studied the spin trapping properties of *N*-2-(2-ethoxycarbonyl-propyl)-α-phenylnitrone (EPPN)[41] bearing an ethoxycarbonyl group in *β*-position.[42], [43] EPPN showed higher lipophilicity than PPN with octanol-phosphate buffer partition coefficient (K_p) of 29.8 and 10.2, respectively, suggesting that EPPN could cross biomembranes. EPPN was also found to be a very efficient trap for carbon-centered radicals but unsuitable for hydroxyl trapping in aqueous conditions. The superoxide spin adduct of EPPN was found to be more stable than those of PBN, DMPO, 4-PyOBN and PPN, yielding to a persistent spin adduct without any artifactual signals.[43] But the short-lived superoxide spin adduct of EPPN at physiological pH has limited its used in biological media. Finally, it was demonstrated that the presence of an electron-withdrawing group at the *β*-position of the nitrone function, in the case of PPN, 4-PyOPN and EPPN, greatly enhances the superoxide adduct stability. Stolze and co-workers have developed EPPN analogs either by replacing the ethoxy group by a propoxy, *iso*-propoxy, *n*-butoxy, *sec*-butoxy and *tert*-butoxy moiety or the phenyl ring by a pyridyl ring (Figure 1.4).[44] As observed for the parent EPPN, these new compounds were efficient for the detection of carbon-centered radical but no significant improvement of the superoxide adduct stability could be obtained for these lipophilic EPPN derivatives.

Two ester nitrone derivatives with improved spin adduct stability were next designed. EPPyON derives from 4-PyOBN by addition of an ethoxycarbonyl group in *β*-position, the oxidopyridinium group exhibiting stabilization of the superoxide spin adduct, whereas DEEPN, bears two electron-withdrawing groups in *β*-position of the nitrone function (Figure 1.4).[42] DEEPN (K_p = 4.8) is less lipophilic than PBN, PPN and EPPN but more lipophilic

than DEPMPO (K_p = 0.16). On the contrary, EPPyON is highly hydrophilic with a partition coefficient of K_p = 0.33. The two nitrones were found to efficiently trap various carbon- and oxygen-centered radicals in aqueous media but were unsuitable for detecting hydroxyl radical. The superoxide spin adducts of these two nitrones decay approximately at the same rate at neutral pH but DEEPN-O_2H spectrum is simpler, making DEEPN an efficient spin trap for superoxide detection in the PBN series.

Consequently, these results led to combine in the same molecule an oxidopyridinium and two ethoxycarbonyl groups yielding the nitrone DEEPyON.[45] Spin trapping abilities were studied as well as the kinetic aspects of the superoxide detection that is the trapping reaction rate constants and for the spin adduct decay at pH 7.2. Results were compared to the monoester nitrone EPPyON and to its corresponding cyclic derivatives bearing two ester groups denoted as DEPO. Unfortunately, it was shown that the presence of a second ester group gave weaker superoxide trapping capacities in the oxidopyridinium series. On the contrary in the cyclic series an opposite effect was observed with DEPO being three times more potent than its corresponding analog with only one ester group (EMPO). However, DEPO-O_2H decayed much faster than EMPO-O_2H at nitrone concentration up to 0.005 mol.L^{-1}, making EMPO preferred in detecting superoxide radical.

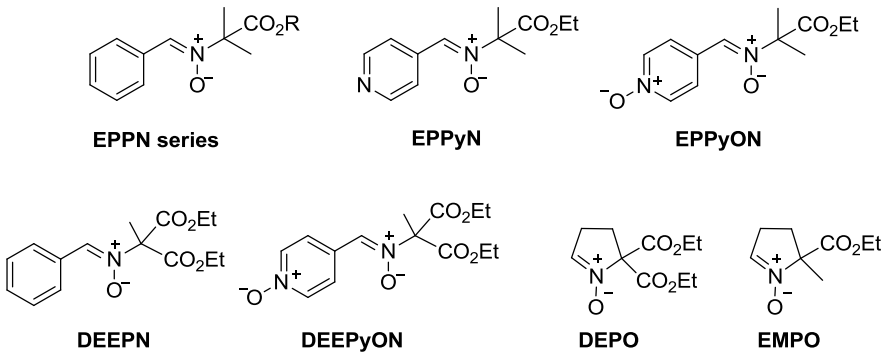


Figure 1.4. EPPN (R = CO_2Et) and the EPPN series (with R = propoxy, *iso*-propoxy, *n*-butoxy, *sec*-butoxy or *tert*-butoxy) as well as other ester derivatives including cyclic nitrones.

***N-tert*-butyl substituted derivatives.** In our group, a series of mono-, di- and tri-*N-tert*-butyl-substituted PBN derivatives was developed in order to study the effects of the substituents in *β*-position of the nitronyl function (Figure 1.5).[46] An increased positivity on the nitronyl carbon was observed and was found to correlate well with experimental NMR chemical shifts. A moderate nucleophilic nature was observed for superoxide addition to nitrone as well as for phenyl radical addition to nitrone using UV-vis. stopped-flow kinetic and spin-trapping kinetics, respectively. Moreover, the antioxidant properties against H_2O_2-induced cell death were found to be affected by the nature of the substituents, with a good correlation with the oxidation potentials as determined by cyclic voltammetry. This work is presented in the second chapter of this thesis report.

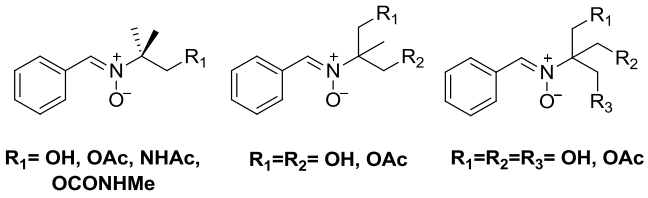


Figure 1.5. mono-, di- and tri-*N-tert*-butyl-substituted PBN derivatives.

Adamantan substituted nitrones. The *tert*-butyl group of PBN was also modified with a cyclic moiety derived from adamantane,[47, 48] which has itself being investigated as bioactive compound. The rational was to increase the lipophilicity of the nitrone and to ensure enhanced spin adduct stability. Hydroxyl adducts exhibited significantly higher stability than those of PBN, very likely due to rigid adamantane ring. Among the adamantly derivatives synthesized, the three following compounds (Figure 1.6) proved soluble enough in water for spin trapping in aqueous media.[48]

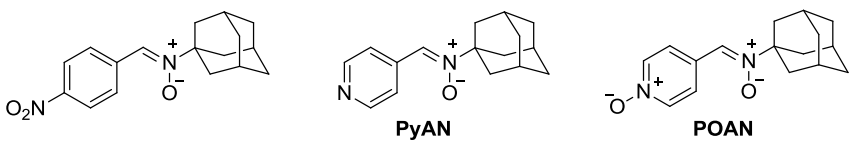


Figure 1.6. Structures of adamantly PBN-type nitrone derivatives.

The hydroxyl adducts stability of PyAN and POAN was the highest of the series and consequently, these two compounds were further study on UV-B-damaged isolated thylakoid membranes. The primary target of UV-B is photosystem (PSII) which is one essential pigment-protein complexes conducting photosynthetic transport. The two adamantyl nitrones were less toxic than their corresponding *N-tert*-butyl nitrones (IC_{50} of ~10 mM for PyAN and POAN compared to 6.7 and 1.9 for PyBN and 4-PyOBN, respectively). Moreover, they proved suitable for detecting hydroxyl radical produced in thylakoid membrane under UV-B stress, with enhanced spin adduct stability.[47]

α-aryl substitutions of PBN. A phosphorylated nitrone derivative bearing a hydroxyl in *para* position of the phenyl ring (4-HOPPN) was found to trap various carbon-, oxygen- and sulfur-centered radicals and to stabilize superoxide spin adduct.[49] The optimized geometry observed for 4-HOPPN superoxide spin adduct confirmed that the introduction of the phosphoryl group efficiently stabilize the spin adduct compare to PBN superoxide spin adduct. Moreover, stronger intra-molecular interactions, including Hydrogen bonding and the nonbonding attractive interactions as well as the bulky steric protection in 4-HOPPN-OOH were suggested to be responsible for stabilizing the superoxide spin-adduct. In the series of *ortho*-, *meta*- and *para*-substituted PBN developed by Janzen and co-workers in the 90s, NMR and EPR data of the *meta*- and *para*-substituted derivatives were found to correlate with the Hammett constants which indicates the polar effect of substituents.[19] Our group has recently worked on the reactivity of six *para*-substituted PBN derivatives toward superoxide radical anion and hydroperoxyl radical, structures are represented in Figure 1.7.[50] Competitive spin trapping using stopped-flow kinetics and EPR spectroscopy were employed to determine the rate of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ radical addition. Computational methods were used to predict the nitronyl atom charge densities and the free energies of superoxide and hydroperoxide radical addition to nitrones. The combined experimental and theoretical data

suggest that the addition of $O_2^{\bullet-}$ is weakly electrophilic and not affected by the substituents properties whereas $HO_2^{\bullet}$ addition is predominantly electrophilic and follows Hammett's equation. The relative rate of $Ph^{\bullet}$ trapping showed no correlation with the Hammett values, indicating the absence of a polar effect and supporting the non nucleophilic nature of phenyl radical. This demonstrated that the reactivity of the nitrone function can be modulated by the nature of the substituents but this is quite dependent to the radical trapped. Moreover, a good correlation was observed between the electrochemical potentials and the Hammett sigma *para* constant (σ_p) with compounds bearing an electron-donating group being more easily oxidized than those bearing an electron-withdrawing group. The polar effect can be explained by the stabilization of the intermediate nitroxide formed through resonance structures. This work is presented in the second chapter of this thesis report.

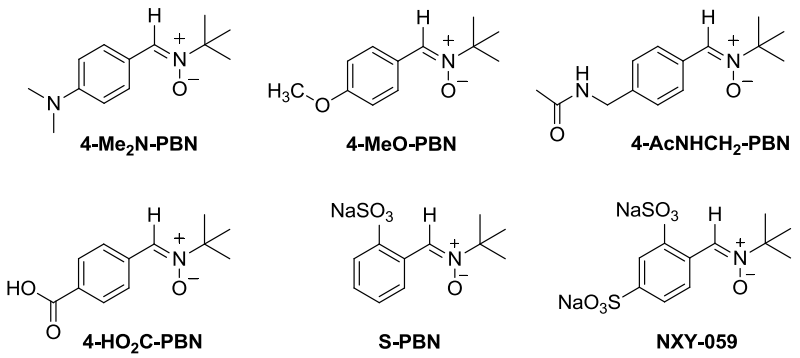


Figure 1.7. Examples of *para*-substituted PBN derivatives including S-PBN and NXY-059.

Sulfophenyl substituted PBN. In order to improve the hydrophilicity of PBN, sulfonic acid substituents were grafted onto the phenyl ring. The *ortho* monosubstituded derivative is called S-PBN and the *ortho*, *para* disubstituted derivative is called NXY-059 (Figure 1.7). The ability of PBN, S-PBN and NYX-059 to form radical adducts and prevent salycilate oxidation were studied in aqueous media.[51] The three compounds were found to efficiently trap oxygen- and carbon-centered radicals and PBN was the most potent to prevent salycilate

oxidation. Yang and co-workers studied the neuroprotective effect of PBN and S-PBN in a focal cerebral ischemia model and a significant attenuation of about 35% infarct volume was noted.[52] This was further confirmed on an embolic model of focal cerebral ischemia[53] and despite differences in their pharmacokinetics and absorption through the blood–brain barrier, the two spin traps appeared to have similar properties. S-PBN also exhibited neuroprotective properties in ischemia/reperfusion nerve injury.[54] However, PBN was found most potent than S-PBN as neuroprotectant in cortical areas, probably due to the poor brain penetration by S-PBN.[55] With two sulfonic acid groups, NXY-059 is more water-soluble but its superiority compared to PBN as a neuroprotectant against stroke was demonstrated.[56] NXY-059 was the first compound to reach phase III clinical trials in the USA conducted by its licensee AstraZeneca.[57, 58] Unfortunately pooled analysis concluded that NXY-059 was not effective,[59] which led to the termination of NXY-059 as therapeutics for acute ischemic stroke. The success of NXY-059 has been mainly limited by its poor bioavailability, limited stability which required administration of high doses. In addition several critiques about the preclinical studies have been raised and it appears that more rigorous and strenuous testing at the preclinical stage is needed when evaluating neuroprotective agents.[60]

Cyclic analogs of PBN. A high interest in developing treatment for stroke, septic shock and related diseases led Hoechst Marion Roussel Int. to develop 3,4-dihydro-3,3-dimethyl-isoquinoline *N*-oxide, a cyclized version of PBN also called MDL 101.02.[61] This compound which was supposed to have more potent radical trap due to the constrained and potentially more coplanar orientation of the nitrone and aromatic ring was in fact less planar than PBN, according to subsequent molecular modeling studies. However, MDL 101.002 was found to be more potent than PBN in several tests of antioxidant and radical trapping activity. A wide range of isoquinoline analogs were next synthesized such as aryl-substituted nitrones as well

as related spiro compounds (Figure 1.8) and they were found to be at least 10-fold better than PBN in reducing liposomes oxidation. Other chemical modifications were conducted with the fused phenyl ring being replaced by a more extended aromatic system or with electron rich heterocycles as well as five, six or seven atom nitrone-containing rings as shown in Figure 1.8.[62, 63]

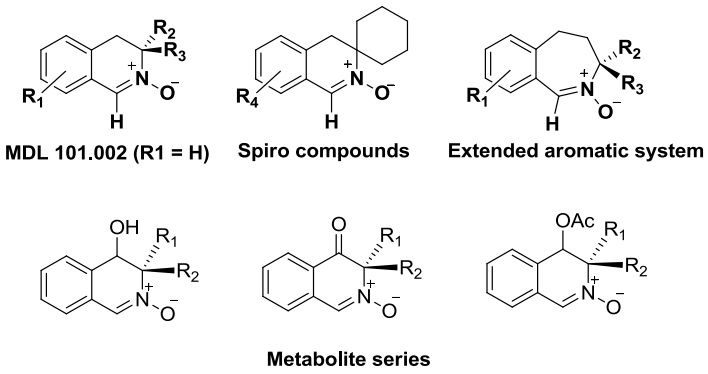


Figure 1.8. General structures of cyclic PBN derivatives including the hydroxylated metabolites and two derivatives.

When a second antioxidant functionality was added such as (*o*,*o'*-dimethylphenol) which is an antioxidant based on α-tocopherol, the activity was increased. The lipophilicity (log k'_W) was measured by a chromatographic technique and a general trend was noted with the more lipophilic compounds being the more active *in vitro*. An interesting hydroxylated metabolite of MDL 101.002 was isolated and identified by the same group.[64] Despite its less potent antioxidant activity than MDL 101.002 in an *in vitro* lipid peroxidation assay, this metabolite was found to greatly reduce acute toxicity and exhibited sedative properties. Therefore, additional analogs were synthesized to increase the lipophilicity of this metabolite. Among them, oxidation and acetylation of the hydroxyl function can be quoted (Figure 1.8), as well as the replacement of the gem-dimethyl moiety with a spirocyclo alkyl group in both alcohol and ketone series. These compounds were found to be more lipophilic and more active than the hydroxyl metabolite in the *in vitro* lipid peroxidation assay, while only the ketone

derivatives were more potent in the *in vitro* neuronal peroxidation assays. This beneficial activity results from the ketone formation which simultaneously increases lipophilicity and removes the stereocenter. In addition, the introduction of an electron-withdrawing group conjugated with the nitrone group renders it more reactive toward radicals. However as regards to the side effects, the improvement brought by the ketones derivatives compared to MDL 101.002 remained limited.

The therapeutic potential of a series of 2-benzazepine nitrones developed by Soto-Otero and co-workers against age-related neurodegenerative disorders was studied.[65, 66] Among the several analogs synthesized, two derivatives bearing respectively a *C*-3 spiro cyclopentyl and a tetrahydropyranyl moieties (Figure 1.9), showed promising protection of dopaminergic neurons intoxicated with 6-hydroxydopamine, a toxin implicated in Parkinson's disease. One of these compounds was also found potent in a model of Alzheimer neurodegeneration. These observations may contribute to improve therapeutic potential of 2-benzazepine nitrones in age-related neurodegenerative diseases.

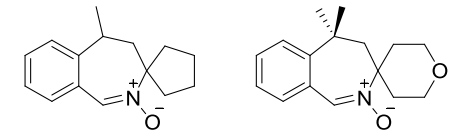


Figure 1.9. Potential neuroprotective agents in age-related diseases.

2. Modification of the nitronyl function

Heteroaryl nitrones. A survey of the literature on heteroaryl nitrones from 1980 to 1999 has been reviewed by Golstein and Lestage, in which pyridyl, thienyl, furyl and immidazolyl substituted nitrones were described.[67] Results have demonstrated that the presence of a hetero-aromatic substituent on the nitrone double bond increased efficiently the stability of the spin-adduct and the ability to trap radicals. Moreover, lipophilicity and solubility are

modulated by the presence of a heterocycle and therefore so is crossing of biological membranes. Several groups have next designed a number of heteroaryl nitrones and their protective effects against various damages were studied. Nepveu and co-workers designed potent compounds against microvascular damages induced by ischemia/reperfusion[68] and a majority of these compounds were more efficient to trap $^{\bullet}CH_3$ than $^{\bullet}OH$ radical. No correlation was observed between the protective effect and their partition coefficient or their capacity to trap $^{\bullet}CH_3$ and $^{\bullet}OH$ radicals. Three compounds derived from piperol, O-benzyl vanillin and furfural (Figure 1.10) were the most potent of the series. Dias *et al*. synthesized other derivatives including *N*-methyl nitrone derivatives, which potency in preventing microvascular damage induced by ischemia/reperfusion.[69] It has to be underlined that a correlation between the lipophilicity and and their biological action was noted.

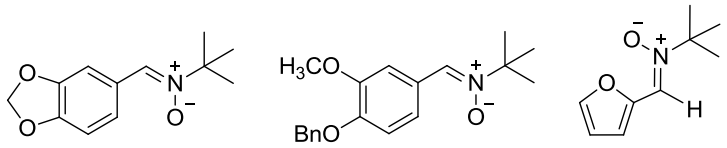


Figure 1.10. Nitrones derived from piperonal, O-benzyl vanillin and furfural.

Porcal and co-workers have synthesized thiadiazole and furanoxyl nitrone derivatives combining in their structures a nitronyl group with neuroprotection properties, an antioxidant fragment and an heterocyclic group able to stabilize the spin adduct (Figure 1.11).[70] These compounds proved excellent spin-trapping capacity, scavenging oxygen, carbon, sulfur and nitrogen-center free radicals.[71] The neuroprotective activity was demonstrated on a human neuronal-like cellular system exposed to H_2O_2 without any cytotoxicity at 10µM. *N*-alkyl substitutions such as *tert*-butyl, cyclohexyl or benzyl group showed to affect both the spin-adduct stability and the biological activity with the *N*-cyclohexyl derivative exhibiting better cell viability than its *N*-*tert*-butyl derivative, while this latter exhibited better radical scavenging activity and the benzyl derivative was the less potent compound. These results

suggest that heteroaryl nitrones have therapeutic potential as neuroprotective agents in preventing cell death against oxidative stress and damage.

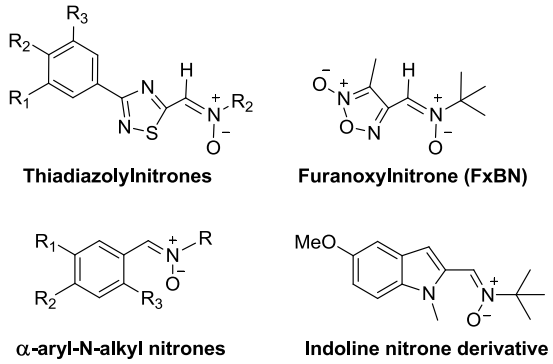


Figure 1.11. General structures of thiadiazolylnitrones, furoxanylnitrones, α-aryl-*N*-alkyl and indoline nitrones.

The 4-furoxanyl nitrone denoted FxBN[71] showed appropriate solubility in aqueous conditions, which is an important physical chemical property for biological application. FxBN formed hydroxyl and superoxide spin adduct with long half-life of >2h and ~0.5 h, respectively, which suggests it could be a better trap than PBN and DMPO for oxygen-trapping in biological conditions.

Samadi and co-worker developed a series of α-aryl and *N*-alkyl substituted heteroaryl nitrones as potential agents for stroke treatment in cerebral ischemia (Figure 1.11). In a first report, various α-aryl-*N*-alkyl nitrones were synthesized in order to evaluate the effect of different electron-donating or electron-withdrawing substituents in the aromatic ring.[72] The *in vitro* antioxidant activity was determined using two different tests. The stable free radical 2,2-diphenyl-1-picylhydrazyl (DPPH) was used as reagent to investigate the scavenger properties and showed that for phenol derivatives, the *para* position was preferred over the *meta* position, as well as the absence of the bromine atom at the *ortho* position. The oxygen radical absorbance capacity (ORAC) using a fluorescent probe was used to measure the peroxyl free radical scavenging ability of nitrones in the second *in vitro* assay. A significant antioxidant

activity was demonstrated for most of the nitrones and the substituted indole was the most potent, followed by the two phenol benzene-type nitrones. Conversely to the first *in vitro* assay, the *meta* position of the hydroxyl group as well as the presence of a bromine in *ortho* position were found to increase the antioxidant activity. PBN was poorly active in the first assay and inactive in the second one. Furthermore, the scavenging of hydroxyl radical was investigated and all the nitrones were able to efficiently trap $^{\bullet}OH$ free radical. Theoretical and computational approaches were used to predict the antioxidant capacity and were found in agreement with the DPPH assay with the phenol derivatives being more efficient in electron transfer processes. The calculated energy values for hydroxyl and peroxyl trapping predicted that the most favorable adduct-spin will take place between the indole derivative and compounds containing a *ortho*-bromo, a *meta*-hydroxyl and a *para*-methoxy function on the aryl group. Finally, the *in vitro* pharmacological analysis was carried out, in human neuroblastoma cells previously stressed, in order to determine the neuroprotective capacity of these nitrones but protection was in general low.

A series of *α*-aryl and heteroaryl-*N*-alkyl nitrones were developed as potential agents for stroke treatment. The *α*-aryl-*N*-alkyl nitrone derivatives comprised a strong electron withdrawing group or a highly hindered phenol or nitrooxy-alkoxy chain at position C-4.[73] The heterocyclic groups, such as quinoline or furan, were substituted with halogen atoms in appropriate position, as showed in Figure 1.12. Theoretical calculations were first carried out to predict their ability to cross the blood-brain-barrier (BBB) and suggested that, except nitrones bearing a 3-(nitrooxy)-alkoxy-benzilidene substituents, they should present a good brain penetration profile. The neuroprotection afforded by the compounds was estimated following Goldstein *et al*. approach which is based on the calculation of orbital energies and lipophilicity,[74] suggesting that nitrones bearing a *para*-hydroxyl group or quinolinyl group would exhibit good level of neuroprotection. On the contrary, the presence of a 3-(nitrooxy)-

alkoxy-benzilidene group would lead to a poor level of neuroprotection. Data showed that both the presence of an electron-withdrawing group (CF_3) in the benzene ring or the quinoline heterocyclic ring system conjugated with the nitrone moiety are key structural motif for neuroprotection. However, the presence of a highly hindered phenol moiety or a bromofuran ring was associated with strong toxicity. Therefore, it demonstrates that theoretical prediction were in good agreement for a majority of the compounds but not for all. It was also observed that *N*-benzyl nitrones presented higher neuroprotection than their corresponding *N*-*tert*-butyl nitrones. Among the whole range of nitrones synthesized, the quinolinyl nitrones showed potent combined antioxidant and neuroprotective properties and could therefore be considered as lead compounds.

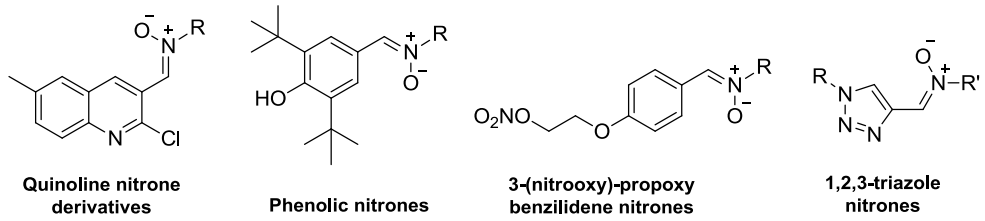


Figure 1.12. Exemples of *a*-aryl, heteroaryl and triazole-*N*-alkyl nitrone derivatives.

More recently, Sambasiva Rao *et al*. developed a series of 1,2,3-triazole substituted *N*-phenyl nitrones and studied their anti-inflammatory and anti-cancer activity against various cancer cells lines.[75] Among the series, only the derivative bearing a six-carbon fluorinated chain exhibited both anti-inflammatory and anti-cancer activity. Most of the others exhibited either good anti-inflammatory or good anti-cancer activity. 4-fluorophenyl or 4-chlorophenyl substitution on the triazole moiety as well as phenyl and *tert*-butyl *N*-alkyl nitrones promoted the highest cytotoxicity which, however, remained significantly lower than doxorubicin. **Imidazolyl nitrones.** A series of imidazolyl nitrones was developed by Servier and exhibited good water-solubility with improved spin-trapping properties than the parent PBN.[74] Imidazolyl nitrones either with aromatic or heteroaromatic cycle substitutions proved higher

efficiency than PBN in an *in vitro* lipid peroxidation test on cortical membranes. Their capacity to oppose lethality induced by intracerebroventricular administration of *tert*-butyl hydroperoxide (*t*-BHP) in mice was also demonstrated with better protection compared to PBN. However, this was accompanied by hypothermia and only the phenylimidazolyl nitrone (Figure 1.13) showed significant protection (80% survival) without any hypothermia observed. The neuroprotection afforded by imidazolyl nitrones was found to correlate with the partition coefficient with the more lipophilic compounds being the more active. Moreover, it was shown that HOMO energy level of imidazolyl nitrones also influenced the biological behavior with higher protection when the HOMO energy is higher.

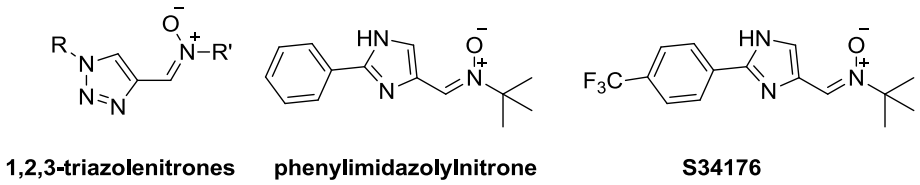


Figure 1.13. Structure of triazole of two imidazolyl nitrones.

The capacity of ten imidazolyl nitrones to directly scavenge free radicals was also studied *in vitro* by Reybier and co-workers and showed a high capacity to trap and stabilize carbon-centered radicals.[76] Among the derivatives, S34176 bearing a trifluoro group in *para* position of the phenylimidazolyl nitrone (Figure 1.13) was selected for its *in vivo* activities. Lockhart and co-workers studied the neuroprotective effect of this compound in different *in vivo* paradigms of neuronal degeneration.[77] When S34176 was administrated 30 min before global ischemia (75mg/kg *i.p.*), the neuronal protection was 50% better compared to the control. Similar injection 5 min after ischemia showed same results. However, when S34176 was administered 3 h post-ischemia, a limited efficacy was observed and this derivative was also ineffective in preventing focal permanent-occlusion ischemia. Unfortunately, the short active window of S34176 demonstrated that this compound alone have a limited therapeutic benefit in acute phase of cerebral ischemia.

Azulenyl and stilbazulenyl nitrones. Since the beginning of the 90s, the Becker's group in a collaborative work with UpJohn Company has been interested in azulenyl nitrones derivatives named AZN (Figure 1.14). As already observed for cyclic analogs and imidazolyl nitrones, the aromatic rings confer higher rigidity and stability to the spin adducts. These compounds are prepared from guaiazulene, a natural product which is itself known to exhibit antioxidant and anti-inflammatory properties[78] and which has been used in a number of medical applications.[79, 80] Another important feature is that the guaiazulene ring system confers an oxidation potential exceptionally low in addition of being lipophilic. Indeed, cyclic voltammetry studies showed that the oxidation potential of AZN was 0.84V *vs*. SCE while that of PBN is 1.47 V. This potential is close to that of essential antioxidants such as glutathione (0.69 V *vs*. SCE) and *β*-carotene (0.76 V *vs*. SCE).[81] Thus, compared to conventional nitrones, azulene-based spin traps exhibit improved antioxidant properties and may more readily penetrate the blood–brain barrier.

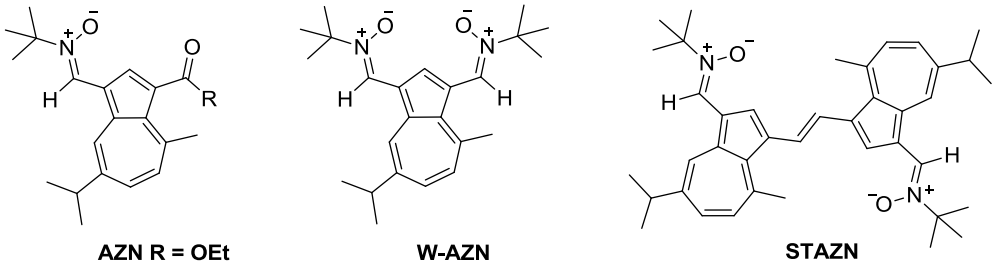


Figure 1.14. Structures of AZN, w-AZN and STAZN.

AZN nitrone also allows a colorimetric approach to the detection, isolation and analysis of free radical adducts in the case of rapid nitroxide decay. Indeed, AZN was found to react with peroxyl radicals to form a nitroxide that decomposes into aldehyde and secondary nitroxide by-products which exhibit different color and therefore can be easily distinguished.[82] Biological results have demonstrated that AZN may be promising as a clinical neuroprotectant in ischemic brain injury.[83] Both AZN and its water-soluble derivative w-

AZA produced significant *in vivo* neuroprotection activity in a mouse Parkinson's disease model using MPTP as a neurotoxin.[84, 85]

A second-generation of azulenyl nitrones called stilbazulenyl nitrones (STAZN) were synthesized in five steps from the natural guaiazulene (Figure 1.14).[86] STAZN possesses an oxidative potential of 0.33 V *vs*. SCE which is about half lower than that of AZN and therefore exceptionally lower compared to PBN. Antioxidant assays demonstrated that STAZN possesses remarkable potency as chain-breaking antioxidant and displays a far superior antioxidant profile comparing to PBN and AZN which were studied at concentration double or more than STAZN. Moreover STAZN appears to be more potent than the first-generation nitrone AZN for neuroprotection *in vivo*.[87] Other studies demonstrated that STAZN is also a highly potent cardioprotective agent in acute coronary ischemia, suggesting the potential for clinical benefit in the setting of acute coronary syndromes.[88]

STAZN was also studied in heterogeneous media *i.e*. micelles and liposomes. The rate constant for reaction with peroxyl radicals and a number of radicals trapped were studied and compared to three phenolic antioxidants and showed that the inhibition rate constant for STAZN depends on the reaction medium and the type of initiator.[89] More recently, *in vitro* assays were carried out to evaluate the efficacy and safety of STAZN as a lead compound to treat ischemic stroke.[90] CeeTow analysis was used to determine the acute toxicity profile of the STAZN and results have shown an excellent safety profile. Moreover, a lack of mutagenic activity was observed, indicating that STAZN may have significant potential as a novel neuroprotective agent and should continue to be developed as a lead compound to treat stroke.

Hartley and co-workers have designed a compound combining in its structure a nitrone spin-trap and a phenol antioxidant with a cyclopropane radical clocklike unit (Figure 1.15). The nitrone and the phenol group acts as radical sensors with different selectivity. Very reactive electron-rich radicals are expected to react with nitrone whereas electron-poor species would

react with the phenol moiety. The cyclopropane should open rapidly to give an unstable primary radical which will cyclize onto nitrone to generate a nitroxyl actuator.[91]

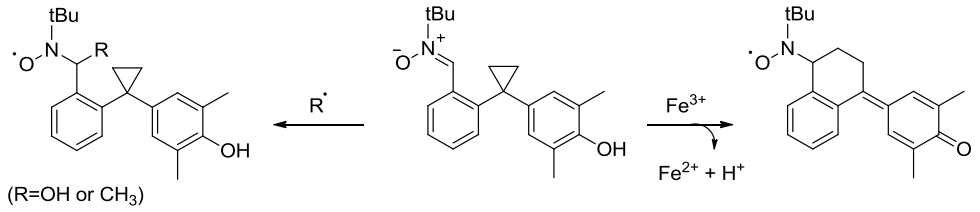


Figure 1.15. Dual detector probe detects and distinguishes between hydroxyl radicals, methyl radicals and iron (III) ions.

3. Towards the targeting of nitrone spin-traps

Although some of the strategies developed towards the synthesis of nitrones with improved antioxidant properties proved to be promising as described above, it appears that one of the limitation in having a "super" nitrone spin-traps results from insufficient control of biodistribution, membrane crossing and specific targeting. Therefore, several groups have designed antioxidants or nitrones derivatives by grafting them onto suitable carriers able to target specific production site of ROS such as the mitochondria or phospholipidic membranes.

Mitochondria-targeting. For example, one of the most promising strategies consists to directly link the antioxidant onto a mitochondria-targeted compounds as mitochondria is the main source and the first target of reactive oxygen species. Lipophilic phosphonium cations were first used to investigate mitochondrial biology by Vladimir Skulachev and colleagues in the late 1960s.[92] Despite their negative charge, lipophilic cations are relatively lipid-soluble and can pass easily through all biological membranes, including the blood-brain-barrier and into muscle cells and thus reach tissues that are most affected by mitochondrial oxidative damage. Therefore, these compounds are able to cross the mitochondria inner membrane and accumulate within the mitochondria matrix. For this reason, they have proven to be among the most useful probes for the investigation of mitochondria function.[93, 94] Various

mitochondria-targeted antioxidants were synthesized by grafting the triphenylphosphonium cations (TPP) to antioxidants such as α-tocopherol (MitoVitE)[95] and PBN spin-trap (MitoPBN)[96] (Figure 1.16). It has been reported that MitoPBN reaches 2.2-4.0 mM concentration within the mitochondria and blocks the oxygen-induced activation of uncoupling proteins. This concept has been extended to cyclic nitrone[97, 98] and to acyclic nitroxide.[99] However, the toxicity of triphenyl-phosphonium derivatives at low concentration has limited their use as therapeutic agents. *N*-aryl pyridinium nitrone (Figure 1.16) have also been used to detect superoxide production from the mitochondria.[100] Carnitine, which is used in the transport of fatty acids into mitochondria, was also used as potential method of targeting the nitronyl group to mitochondria and the conjugate CarnDOD-7C was prepared (Figure 1.16).[101]

Biological membrane-targeting. Another family of spin traps, tailored to intercept radicals within biological membranes was developed such as the DOD-8C nitrone (Figure 1.16).[102] In lipid membranes, its hydrophilic carboxylate group orientates towards the aqueous phase, whereas the nitrone moiety sunk into the membrane. The radical penetration of lipid bilayers was determined using lipophilic spin traps with a combination of NMR and ESR techniques. Three families of homologous spin-traps derives from DMPO, PBN and adamantyl-PBN were synthesized by varying the chain length of the substituent and therefore the overall lipophilicity.[103] The intercalation depth of these spin-traps within the liposomal bilayer was then determined, in order to predict the susceptibility of lipid moieties to radicals attack. This showed that the more lipophilic the spin adduct, the deeper it is found in the bilayer. However, the depth of penetration also depends on the steric bulk of the intercalant.[104] Lipophilic nitrones with two long alkyl chains have also been developed[105, 106] as well as a lipophilic β-cyclodextrin cyclic nitrone conjugate[107] or cholesteryl-based nitrones.[108, 109]

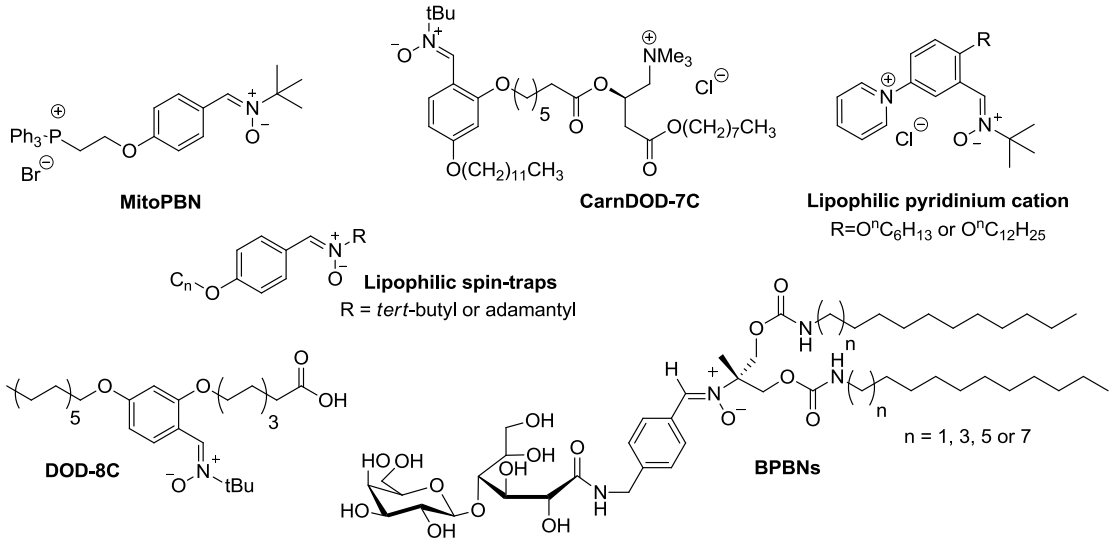


Figure 1.16. Examples of lipophilic cationic and neutral nitrones.

Amphiphilic-nitrone derivatives.

With the expectation that amphiphilic compounds possessing both a hydrophilic polar head and a lipophilic group would exhibit improved bioavailability and membrane crossing ability, our work over the past 15 years has been devoted to the design and the synthesis of amphiphilic antioxidants with a particular attention to nitrones derivatives. The use of antioxidants is limited by their low bioavailability and conjugation to an amphiphilic carrier is expected to improve the bioactivity. Tuning of the hydrophilic/lipophilic balance would ensure a better ability to cross membranes and consequently would improve the protective activity of nitrone-type synthetic antioxidants. The lipophilic group is typically a large hydrocarbon or fluorocarbon moiety and confers to the molecule a hydrophobic character; while the hydrophilic polar functional group can be a charged group such as anionic or cationic groups or an uncharged group. As a result, an amphiphilic compounds which has lipophilic and hydrophilic groups may dissolve in water and to some extent in non-polar organic solvents.

Our laboratory has focused on the design of amphiphilic nitrone compounds. Two original series of amphiphilic PBN derivatives were developed. In the first series, a polar head group was grafted onto the aromatic ring of PBN and a hydrophobic moiety, either hydrogenated or perfluorinated, was linked to the *N*-*tert*-butyl group through a thioether, a carbamate or an amide bond.[110] The nature and the length of the hydrophobic chain as well as the nature of the polar head were varied,[105, 111, 112] and some examples are represented in Figure 1.17. Preliminary biological evaluations showed that amphiphilic derivatives were more potent than PBN, demonstrating that amphiphilicity is a key feature in determining bioactivity and protection against *in vitro* and *in vivo* oxidative toxicity.[110, 111, 113, 114] Among the series, LPBNAH (Figure 1.17) exhibited very high antioxidant activity as well as anti-ageing effect.[112, 115, 116] A structural isomer, for which the position of the polar head and hydrophobic chain on the PBN moiety is reversed were next synthesized (Figure 1.17) and showed even better potency in preventing oxidative-stress mediated damages.[117] However, this first series was only developed for the PBN moiety. In order to extend the amphiphilic strategy to other antioxidants, we, therefore, developed a second series of amphiphilic carriers.

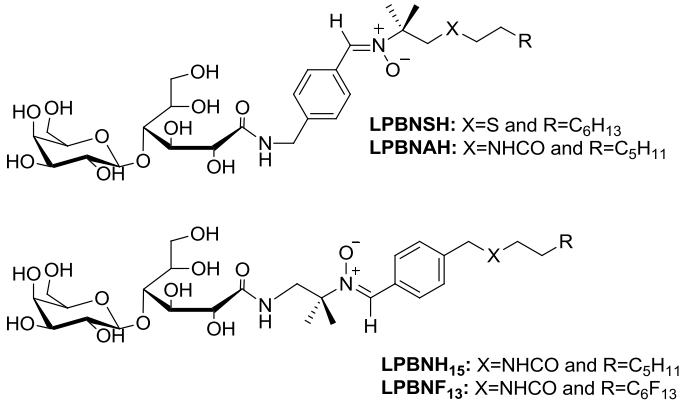


Figure 1.17. Examples of the first series of amphiphilic PBN derivatives.

In the second series, an amino acid was used as the core of the carrier upon which hydrophobic and hydrophilic parts are linked, respectively by the carboxyl and amino groups

of the amino acid. The choice of an amino acid as a central scaffold was evident both for its absence of toxicity and for the various functionalization possibilities it allows. Among the twenty amino acids available, lysine (bearing an amino group) and aspartic acid (bearing a carboxylic acid group) were chosen because they can be easily functionalized on their side chains, through an amide bond. The carrier was made of : *i*) a glycosidic polar head derived from lactobionic acid which provides good water solubility to the molecule, *ii*) a perfluorinated chain that supplies hydrophobicity without inducing cytolytic effect[118] and therefore enabling membrane crossing ability, and *iii*) an amino acid as scaffold, bridging the polar and apolar groups, and upon which the antioxidant moiety can be grafted through an amine bond. The choice of the antioxidants was supported by their singular intrinsic properties. In a first study, the biological activity of three antioxidants such as lipoic acid, indole-3-propionic acid and Trolox® as well as PBN were studied (Figure 1.18).[119] Using *in vitro* primary cortical mixed cell cultures and *in vivo* rotifers cultures we confirmed that the amphiphilic character of the antioxidant drug improve the protection by at least two times. Only for indole-3-propionic acid, the protection was lowered after conjugation to the amphiphilic carrier indicating that the free acid group may be involve in the antioxidant mechanisms.

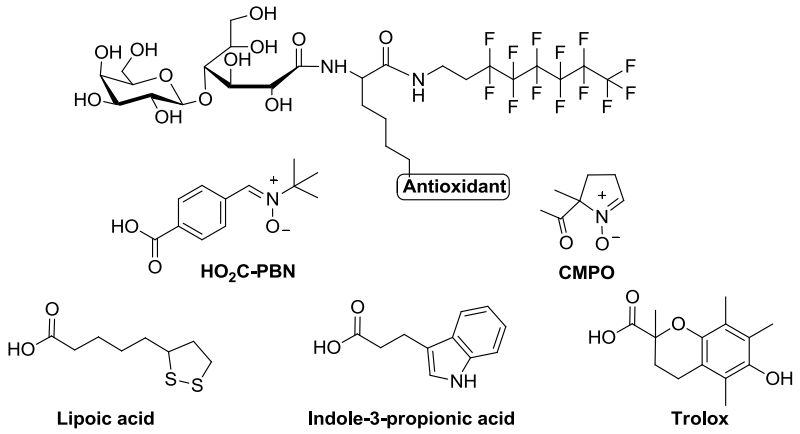


Figure 1.18. Examples of the second series of amphiphilic antioxidant derivatives including the PBN derivative called FAPBN and the DMPO derivative called FAMPO.

More recently, the cyclic nitrone DMPO was grafted onto the same amphiphilic carrier (FAMPO) and its protection against different stress inducers was compared to the PBN derivative (FAPBN) and to the non-amphiphilic parent compounds.[120] Cytoprotection was studied on bovine aortic endothelial cells (BAEC) against hydrogen peroxide (H_2O_2), 3-morpholinosynonimine hydrochloride (SIN-1) and 4-hydroxynonenal (HNE) induced cell-death. FAPBN was found to be the most efficient against H_2O_2 whereas FAMPO was better protective against $ONOO^-$ and none of them were effective against HNE. This indicates that all amphiphilic nitrones were more potent than their corresponding parent derivatives. However, the difference in the cytoprotective properties of FAPBN and FAMPO may indicate different intrinsic antioxidant properties and localization in the cell. The continuation of this work will be presented in the third chapter of this thesis report. I will describe a series of derivatives in which two lysine amino acids have been conjugated allowing the presence of two antioxidant groups on the same amphiphilic. Along the series, we will tune the hydrophilic/lipophilic balance of the derivatives from lipophilic ones (without a polar head) to amphiphilic ones (with a lactobionamide polar head group). The second part of the chapter will present the latest series developed in which the polar head group is a specific ligand that targets melanosomes. Physical-chemical and antioxidant properties of these new amphiphilic nitrone derivatives will be presented as well.

Conclusion

Nitrone spin traps have been recognized as efficient protecting agent against harmful oxidative stress in *in vitro* and *in vivo* models and therefore they can both be used as analytical and biological tools. The connectivity as well as the nature and the position of substituents on a nitrone molecule may significantly affect the chemical and biological properties of the nitronyl group. Over the past years, research has been notably focused on the development of analogs of both PBN and DMPO in order to improve their biological and

spin-trapping properties. Among the PBN derivatives described in this chapter, simple chemical structural modifications were firstly carried out either on the phenyl ring or on the *N-tert*-butyl group of PBN. Various analogs were designed to improve the spin-adduct stability, we can cite the addition of a phosphorylated substituent on the *N-tert*-butyl group of the nitronyl which led to increased stability of the spin adducts formed. Furthermore, cyclic PBN analogs as well as heteroaryl or azulenyl nitrone derivatives were found to increase the spin-adduct stability by the presence of an aromatic ring which confers higher rigidity to the system. From the therapeutic side, of particular interest is the sulfophenyl series which went up to phase III clinical trials although their spin-trapping activity was lower than PBN. This clearly confirms previous findings that the biological protection afforded by PBN-type nitrone is not mediated by the spin-trapping action. In some series, a second antioxidant moiety was added in the nitrone structure such as a guaiazulene or phenolic groups. Through these various chemical modification, the intrinsic properties of nitrones has been improved in some cases but at the moment no nitrone derivative used as a drug is on the market.

Another strategy to improve the bioactivity of nitrone has been focused on improving its bioavailability. Conjugation to a lipophilic cations has shown to specifically target the mitochondria allowing detection of free radical on site. Another approach relies on the use of amphiphilic carriers which are believed to improve the ability to cross cell membranes. By modulating the hydrophilic/lipophilic balance of the cargos, several amphiphilic derivatives were developed in our group and showed high potency in preventing oxidative stress mediated damages in *in vitro* and *in vivo* models.

References

[1] T. Yoshikawa, Y. Naito, What Is Oxidative Stress?, J. Japan Med Ass., 45 (2002) 271-276.

[2] J. Li, W. O, W. Li, Z.-G. Jiang, H. Ghanbari, Oxidative Stress and Neurodegenerative Disorders, Int. J. Mol. Sci., 14 (2013) 24438-24475.

[3] The Effect of Vitamin E and Beta Carotene on the Incidence of Lung Cancer and Other Cancers in Male Smokers, New England Journal of Medicine, 330 (1994) 1029-1035.

[4] A. Ascherio, E.B. Rimm, M.A. Hernain, E. Giovannucci, I. Kawachi, M.J. Stampfer, W.C. Willett, Relation of Consumption of Vitamin E, Vitamin C, and Carotenoids to Risk for Stroke among Men in the United States, Annals of Internal Medicine, 130 (1999) 963-970.

[5] L. Kritharides, R. Stocker, The use of antioxidant supplements in coronary heart disease, Atherosclerosis, 164 (2002) 211-219.

[6] D.G. Meyers, P.A. Maloley, D. Weeks, Safety of antioxidant vitamins, Arch. Internal Medicine, 156 (1996) 925-935.

[7] F.A. Villamena, J.L. Zweier, Detection of reactive oxygen and nitrogen species by EPR spin trapping, Antiox. Red. Signal., 6 (2004) 619-629.

[8] C. Fréjaville, H. Karoui, B. Tuccio, F.L. Moigne, M. Culcasi, S. Pietri, R. Lauricella, P. Tordo, 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: A new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals, J. Med. Chem., 38 (1995) 258-265.

[9] G. Olive, A. Mercier, F. Le Moigne, A. Rockenbauer, P. Tordo, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide: evaluation of the spin trapping properties, Free Radical Biol. Med., 28 (2000) 403-408.

[10] H. Zhao, J. Joseph, H. Zhang, H. Karoui, B. Kalyanaraman, Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals, Free Radical Biol. Med., 31 (2001) 599-606.

[11] F.A. Villamena, A. Rockenbauer, J. Gallucci, M. Velayutham, C.M. Hadad, J.L. Zweier, Spin trapping by 5-carbamoyl-5-methyl-1-pyrroline N-oxide (AMPO): Theoretical and experimental studies, J. Org. Chem., 69 (2004) 7994-8004.

[12] F.A. Villamena, S. Xia, J.K. Merle, R. Lauricella, B. Tuccio, C.M. Hadad, J.L. Zweier, Reactivity of superoxide radical anion with cyclic nitrones: Role of intramolecular H-Bond and electrostatic effects, J. Am. Chem. Soc, 129 (2007) 8177-8191.

[13] R.F. Haseloff, K. Mertsch, E. Rohde, I. Baeger, I.A. Grigor'ev, I.E. Blasig, Cytotoxicity of spin trapping compounds, FEBS Letters, 418 (1997) 73-75.

[14] E.G. Janzen, J.L. Poyer, C.F. Schaefer, P.E. Downs, C.M. BuBose, Biological spin trapping II. Toxicity of nitrones spin traps: dose-ranging in the rat, J. Biochem. Biophys. Methods, 30 (1995) 239-247.

[15] C.F. Schaefer, E.G. Janzen, M.S. West, J.L. Poyer, S.D. Kosanke, Blood chemistry changes in the rat Induced by high doses of nitronyl free radicals spin traps, Free Radical Biol. Med., 21 (1996) 427-436.

[16] K.J. Liu, Y. Kotake, M. Lee, M. Miyake, K. Sugden, Z. Yu, H.M. Swartz, High-performance liquid chromatography study of the pharmacokinetics of various spin traps for application to in vivo spin trapping, Free Radical Biol. Med., 27 (1999) 82-89.

[17] G. Chen, T.M. Bray, E.G. Janzen, P.B. McCay, Excretion, metabolism and tissue distribution of a spin trapping agent, a-phenyl-N-tert-butyl-nitrone (PBN) in rats, Free Rad. Res. Comms., 9 (1990) 317-323.

[18] H.-Y. Cheng, T. Liu, G. Feurerstein, F.C. Barone, Distribution of Spin-Trapping Compounds in Rat Blood and Brain: In Vivo Microdialysis Determination Free Radical Biol. Med., 14 (1993) 243-250.

[19] R.D. Hinton, E.G. Janzen, Synthesis and characterization of phenyl-substituted C-phenyl-N-tert-butylnitrones and some of their radical adducts, J. Org. Chem. , 57 (1992) 2646-2651.

[20] G.M. Rosen, B. Britigan, E., H. Halpern, J., S. Pou, Free Radicals: Biology and Detection By Spin Trapping, Oxford University Press, New York, 1999.

[21] Y. Ogata, Y. Sawaki, Peracid oxidation of imines. Kinetics and mechanism of competitive formation of nitrones and oxaziranes from cyclic and acyclic imines, J. Am. Chem. Soc., 95 (1973) 4692-4698.

[22] D.R. Boyd, P.B. Coulter, M.R. McGuckin, N.D. Sharma, W.B. Jennings, V.E. Wilson, Imines and derivatives. Part 24. Nitrone synthesis by imine oxidation using either a peroxyacid or dimethyldioxirane, J. Chem. Soc., Perkin Trans. 1, (1990) 301-306.

[23] K. Nakama, S. Seki, S. Kanemasa, A new synthetic access to N-alkylated nitrones through Lewis acid-catalyzed conjugate additions of aldoximes, Tet. Lett., 42 (2001) 6719-6722.

[24] S.K. Hota, A. Chatterjee, P.K. Bhattacharya, P. Chattopadhyay, A green chemical approach for the N-alkylation of aldoximes to form nitrones in organized aqueous media and their in situcycloaddition with olefins, Green Chem., 11 (2009) 169-176.

[25] G.P. Novelli, P. Angiolini, R. Tani, G. Consales, L. Bordi, Phenyl-tert-butylnitrone is active against traumatic shock in rats, Free Rad. Res. Comm., 1 (1986) 321-327.

[26] R.A. Floyd, R.D. Kopke, C.-H. Choi, S.B. Foster, S. Doblas, R. Towner, A., Nitrones as therapeutics, Free Radical Biol. Med., 45 (2008) 1361-1374.

[27] F.A. Villamena, A. Das, K.M. Nash, Potential implication of the chemical properties and bioactivity of nitrone spin traps for therapeutics, Future Medicinal Chemistry, 4 (2012) 1171-1207.

[28] R.A. Floyd, K. Hensley, M.J. Forster, J.A. Kelleher-Andersson, P.L. Wood, Nitrones, their value as therapeutics and probes to understand aging, Mech. Ageing Dev., 123 (2002) 1021-1031.

[29] E.J. Locigno, J.L. Zweier, F.A. Villamena, Nitric oxide release from the unimolecular decomposition of the superoxide radical anion adduct of cyclic nitrones in aqueous medium, Org. Biomol. Chem., 3 (2005) 3220-3227.

[30] M.D. Croitoru, F. Ibolya, M.C. Pop, T. Dergez, B. Mitroi, M.T. Dogaru, B. Tőkés, Nitrones are able to release nitric oxide in aqueous environment under hydroxyl free radical attack, Nitric Oxide, 25 (2011) 309-315.

[31] K. Hensley, Q.N. Pye, M.L. Maidt, C.A. Stewart, K.A. Robinson, F. Jaffrey, R.A. Floyd, Interaction of α-phenyl-N-tert-butyl nitrone and alternative electron acceptors with complex I indicates a substrate reduction site upstream from the rotenone binding site, J. Neurochem., 71 (1998) 2549-2557.

[32] Y. Kotake, E.G. Janzen, Decay and fate of the hydroxyl radical adduct of .alpha.-phenyl-N-tert-butylnitrone (PBN) in aqueous media, J. Am. Chem. Soc., 113 (1991) 9503-9506.

[33] E.G. Janzen, C.M. DuBose, Y. Kotake, 2,4,6-trimethoxyphenyl tert-butyl nitrone (MO)3PBN, as a detector of hydroxyl radicals in the presence of peroxides, superoxide and peroxyl radicals, Tet. Lett., 31 (1990) 7395-7398.

[34] Y. Abe, S. Shin-Ya, K. Sakakihara, M. Hirota, Spin trapping of oxygen-centred radicals by substituted N-benzylidene-tert-butylamine N-oxides, (1991) 7.

[35] E.G. Janzen, Y. Kotake, H. Randall D, Stabilities of hydroxyl radical spin adducts of PBN-type spin traps, Free Rad. Biol. Med., 12 (1992) 169-173.

[36] C. Frejaville, H. Karoui, B. Tuccio, F.L. Moigne, M. Culcasi, S. Pietri, R. Lauricella, P. Tordo, 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: A New Efficient Phosphorylated Nitrone for the in Vitro and in Vivo Spin Trapping of Oxygen-Centered Radicals, J. Med. Chem., 38 (1995) 258-265.

[37] B. Tuccio, R. Lauricella, C. Frejaville, J.-C. Bouteiller, P. Tordo, Decay of the hydroperoxyl spin adduct of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: an EPR kinetic study, J. Chem. Soc., Perkin Trans. 2, (1995) 295-298.

[38] B. Tuccio, A. Zeghdaoui, J.-P. Finet, V. Cerri, P. Tordo, Use of new beta-phosphorylated nitrones for the spin trapping of free radicals, Res. Chem. Intermed., 22 (1996) 393-404.

[39] A. Zeghdaoui, B. Tuccio, J.-P. Finet, V. Cerri, P. Tordo, b-Phosphorylated a-phenyl-N-tert-butylnitrone (PBN) analogs: A new series of spin traps for oxyl radicals, J. Chem. Soc., Perkin Trans. 2 12 (1995) 2087-2089.

[40] C. Rizzi, S. Marque, F. Belin, J. Bouteiller, C., R. Lauricella, B. Tuccio, V. Cerri, P. Tordo, PPN-type Nitrones : Preparation and Use of a New Series of beta-phosphorylated spin-trapping agents, (1997) 6.

[41] W. Kliegel, G. Lubkowitz, S. Rettig, J. Trotter, Can. J. Chem., 71 (1993) 2129-2138.

[42] A. Allouch, V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, Preparation and use as spin trapping agents of new ester-nitrones, Org. Biomol. Chem., 1 (2003) 593-598.

[43] V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, N-2-(2-Ethoxycarbonyl-propyl) [alpha]-Phenylnitrone: An Efficacious Lipophilic Spin Trap for Superoxide Detection, Archives of Biochemistry and Biophysics, 397 (2002) 51-56.

[44] K. Stolze, N. Udilova, T. Rosenau, A. Hofinger, H. Nohl, Spin trapping of superoxide, alkyl, and lipid-derived radicals with derivatives of the spin trap EPPN, Biochem. Pharmacol., 66 (2003) 1717-1726.

[45] A. Allouch, V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, Spin trapping of superoxide by diester-nitrones, Org. Biomol. Chem., 3 (2005) 2458-2462.

[46] M. Rosselin, F. Choteau, K. Zéamari, K.M. Nash, A. Das, R. Lauricella, E. Lojou, B. Tuccio, F.A. Villamena, G. Durand, Reactivities of Substituted α-Phenyl-N-tert-butyl Nitrones, J. Org. Chem., 79 (2014) 6615-6626.

[47] E. Hideg, A. Takátsy, C.P. Sár, I. Vass, K. Hideg, Utilizing new adamantyl spin traps in studying UV-B-induced oxidative damage of photosystem II, Journal of Photochemistry and Photobiology B: Biology, 48 (1999) 174-179.

[48] C.P. Sár, É. Hideg, I. Vass, K. Hideg, Synthesis of α-aryl N-adamant-1-yl nitrones and using them for spin trapping of hydroxyl radicals, Bioorganic & Medicinal Chemistry Letters, 8 (1998) 379-384.

[49] Y.-P. Liu, L.-F. Wang, Z. Nie, Y.-Q. Ji, Y. Liu, K.-J. Liu, Q. Tian, Effect of the Phosphoryl Substituent in the Linear Nitrone on the Spin Trapping of Superoxide Radical and the Stability of the Superoxide Adduct: Combined Experimental and Theoretical Studies, J. Org. Chem., 71 (2006) 7753-7762.

[50] G. Durand, F. Choteau, B. Pucci, F.A. Villamena, Reactivity of Superoxide Radical Anion and Hydroperoxyl Radical with a-Phenyl-N-tert-butylnitrone (PBN) Derivatives, J. Phys. Chem. A, 112 (2008) 12498-12509.

[51] K.R. Maples, Z. Y.K., Comparison of the radical trapping ability of PBN, S-PPBN and NXY-059, Free Radical Research, 34 (2001) 417-426.

[52] Y. Yang, Q. Li, A. Shuaib, Neuroprotection by 2-h Postischemia Administration of Two Free Radical Scavengers, a-phenyl-N-tert-butyl-nitrone (PBN) and N-tert-butyl-(2-sulfophenyl)-nitrone (S-PBN), in Rats Subjected to Focal Embolic Cerebral Ischemia, Experimental Neurology, 163 (2000) 39-45.

[53] N. Marklund, F. Clausen, T.K. McIntosh, L. Hillered, Free radical scavenger post-treatment improves functional and morphological outcome after fluid percussion injury in the rat, J. Neurotrauma, 19 (2001) 821-832.

[54] C. Gray, H. Nukada, D.M. Jackson, P.D. McMorran, A. Wu, F. Ma, Neuroprotective effects of nitrone radical scavenger S-PBN on reperfusion nerve injury in rats, Brain Research, 982 (2003) 179-185.

[55] S.L. Peterson, R.S. Purvis, J.W. Griffith, Comparison of Neuroprotective Effects Induced by Î±-Phenyl-N-tert-butyl nitrone (PBN) and N-tert-Butyl-Î±-(2 sulfophenyl) nitrone (S-PBN) in Lithium-Pilocarpine Status Epilepticus, NeuroToxicology, 26 (2005) 969-979.

[56] A.R. Green, T. Ashwood, T. Odergren, D.M. Jackson, Nitrones as neuroprotective agents in cerebral ischemia, with particular reference to NXY-059, Pharmacology & Therapeutics, 100 (2003) 195-214.

[57] K.R. Lees, J.A. Zivin, T. Ashwood, A. Davalos, S.M. Davis, H.-C. Diener, J. Grotta, P. Lyden, A. Shuaib, H.-G.r. HÃ¥rdemark, W.W. Wasiewski, NXY-059 for Acute Ischemic Stroke, New Engl. J. Med., 354 (2006) 588-600.

[58] A. Shuaib, K.R. Lees, P. Lyden, J. Grotta, A. Davalos, S.M. Davis, H.-C. Diener, T. Ashwood, W.W. Wasiewski, U. Emeribe, NXY-059 for the treatment of acute ischemic stroke, New Engl. J. Med., 357 (2007) 562-571.

[59] H.-C. Diener, K.R. Lees, P. Lyden, J. Grotta, A. Davalos, S.M. Davis, A. Shuaib, T. Ashwood, W. Wasiewski, V. Alderfer, H.-G. Hårdemark, L. Rodichok, S.I.a.I.I.I. for the, NXY-059 for the treatment of acute stroke: Pooled analysis of the SAINT I and II Trials, Stroke, 39 (2008) 1751-1758.

[60] S.I. Savitz, A critical appraisal of the NXY-059 neuroprotection studies for acute stroke: A need for more rigorous testing of neuroprotective agents in animal models of stroke, Exp. Neurol., 205 (2007) 20-25.

[61] R.C. Bernotas, C.E. Thomas, A.A. Carr, T.R. Nieduzak, G. Adams, D.F. Ohlweiler, D.A. Hay, Synthesis and radical scavenging activity of 3,3-dialkyl-3,4-dihydro-isoquinoline 2-oxides, Bioorg. Med. Chem. Lett., 6 (1996) 1105-1110.

[62] C.E. Thomas, D.F. Ohlweiler, A.A. Carr, T.R. Nieduzak, D.A. Hay, G. Adams, R. Vaz, R.C. Bernotas, Characterization of the Radical Trapping Activity of a Novel Series of Cyclic Nitrone Spin Traps, J. Biol. Chem., 271 (1996) 3097-3104.

[63] C.E. Thomas, D.F. Ohlweiler, V.L. Taylor, C.J. Schmidt, Radical Trapping and Inhibition of Iron-Dependent CNS Damage by Cyclic Nitrone Spin Traps, J. Neurochem., 68 (1997) 1173-1182.

[64] C.E. Thomas, P. Bernardelli, S.M. Bowen, S.F. Chaney, D. Friedrich, D.A. Janowick, B.K. Jones, F.J. Keeley, J.H. Kehne, B. Ketteler, D.F. Ohlweiler, L.A. Paquette, D.J. Robke, T.L. Fevig, Cyclic Nitrone Free Radical Traps: Isolation, Identification, and Synthesis of 3,3-Dimethyl-3,4-dihydroisoquinolin-4-ol N-Oxide, a Metabolite with Reduced Side Effects, J. Med. Chem., 39 (1996) 4997-5004.

[65] R. Soto-Otero, E. Méndez-Álvarez, S. Sánchez-Iglesias, J.L. Labandeira-García, J. Rodríguez-Pallares, F.I. Zubkov, V.P. Zaytsev, L.G. Voskressensky, A.V. Varlamov, M. de Candia, F. Fiorella, C. Altomare, 2-Benzazepine Nitrones Protect Dopaminergic Neurons against 6-Hydroxydopamine-Induced Oxidative Toxicity, Archiv der Pharmazie, 345 (2012) 598-609.

[66] R. Soto-Otero, E. Mendez-Alvarez, S. Sanchez-Iglesias, F.I. Zubkov, L.G. Voskressensky, A.V. Varlamov, M. de Candia, C. Altomare, Inhibition of 6-hydroxydopamine-induced oxidative damage by 4,5-dihydro-3H-2-benzazepine N-oxides, Biochem. Pharmacol., 75 (2008) 1526-1537.

[67] S. Goldstein, P. Lestage, Chemical and Pharmacological Aspects of Heteroaryl-Nitrones, Current Medicinal Chemistry, 7 (2000) 1255-1267.

[68] S. Kim, G.V.M. de A. Vilela, J. Bouajila, A.G. Dias, F.Z.G.A. Cyrino, E. Bouskela, P.R.R. Costa, F. Nepveu, a-Phenyl-N-tert-butyl nitrone (PBN) derivatives: Synthesis and protective action against microvascular damages induced by ischemia/reperfusion, Bioorg. Med. Chem., 15 (2007) 3572-3578.

[69] A.G. Dias, C.E.V. Santos, F.Z.G.A. Cyrino, E. Bouskela, P.R.R. Costa, N-tert-Butyl and N-methyl nitrones derived from aromatic aldehydes inhibit macromolecular permeability increase induced by ischemia/reperfusion in hamsters, Bioorg. Med. Chem., 17 (2009) 3995-3998.

[70] W. Porcal, P. Hernández, M. González, A. Ferreira, C. Olea-Azar, H. Cerecetto, A. Castro, Heteroarylnitrones as Drugs for Neurodegenerative Diseases: Synthesis, Neuroprotective Properties, and Free Radical Scavenger Properties, Journal of Medicinal Chemistry, 51 (2008) 6150-6159.

[71] G. Barriga, C. Olea-Azar, E. Norambuena, A. Castro, W. Porcal, A. Gerpe, M. Gonzalez, H. Cerecetto, New heteroaryl nitrones with spin trap properties: Identification of a 4-furoxanyl derivative with excellent properties to be used in biological systems, Bioorg. Med. Chem., 18 (2010) 795-802.

[72] A. Samadi, E. Soriano, J. Revuelta, C. Valderas, M. Chioua, I. Garrido, B. Bartolomé, I. Tomassolli, L. Ismaili, L. González-Lafuente, M. Villarroya, A.G. García, M.J. Oset-Gasque, J. Marco-Contelles, Synthesis, structure, theoretical and experimental in vitro

antioxidant/pharmacological properties of α-aryl, N-alkyl nitrones, as potential agents for the treatment of cerebral ischemia, Bioorg. Med. Chem., 19 (2011) 951-960.

[73] M. Chioua, D. Sucunza, E. Soriano, D. Hadjipavlou-Litina, A. Alcázar, I. Ayuso, M.J. Oset-Gasque, M.P. González, L. Monjas, M.I. Rodríguez-Franco, J. Marco-Contelles, A. Samadi, α-Aryl-N-alkyl Nitrones, as Potential Agents for Stroke Treatment: Synthesis, Theoretical Calculations, Antioxidant, Anti-inflammatory, Neuroprotective, and Brain–Blood Barrier Permeability Properties, J. Med. Chem., 55 (2011) 153-168.

[74] A. Dhainaut, A. Tizot, E. Raimbaud, B. Lockhart, P. Lestage, S. Goldstein, Synthesis, Structure, and Neuroprotective Properties of Novel Imidazolyl Nitrones, J. Med. Chem., 43 (2000) 2165-2175.

[75] P. Sambasiva Rao, C. Kurumurthy, B. Veeraswamy, G. Santhosh Kumar, Y. Poornachandra, C. Ganesh Kumar, S.B. Vasamsetti, S. Kotamraju, B. Narsaiah, Synthesis of novel 1,2,3-triazole substituted-N-alkyl/aryl nitrone derivatives, their anti-inflammatory and anticancer activity, Eur. J. Med. Chem., 80 (2014) 184-191.

[76] K. Reybier, J. Boyer, V. Farines, F. Camus, J.-P. Souchard, M.-C. Monje, V. Bernardes-Genisson, S. Goldstein, F. Nepveu, Radical trapping properties of imidazolyl nitrones, Free Rad. Res., 40 (2006) 11-20.

[77] B. Lockhart, A. Roger, N. Bonhomme, S. Goldstein, P. Lestage, In vivo neuroprotective effects of the novel imidazolyl nitrone free-radical scavenger (Z)-a-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone (S34176), Eur. J. Pharmacol., 511 (2005) 127-136.

[78] A.P. Kourounakis, E.A. Rekka, P.N. Kourounakis, Antioxidant Activity of Guaiazulene and Protection Against Paracetamol Hepatotoxicity in Rats, J. Pharm. Pharmacol., 49 (1997) 938-942.

[79] A.P. Kourounakis, E.A. Rekka, P.N. Kourounakis, Effect of Guaiazulene on Some Cytochrome P450 Activities. Implication in the Metabolic Activation and Hepatotoxicity of Paracetamol, Archiv der Pharmazie, 330 (1997) 7-11.

[80] T. Gunes, M.A. Akin, D. Sarici, K. Hallac, S. Kurtoglu, T. Hashimoto, Guaiazulene: a new treatment option for recalcitrant diaper dermatitis in NICU patients, Journal of Maternal-Fetal and Neonatal Medicine, 26 (2013) 197-200.

[81] D. A. Becker, R. Natero, L. Echegoyen, R. C. Lawson, Redox behaviour of azulenyl nitrones: fully reversible one electron oxidation by cyclic voltammetry at potentials in the range of biological antioxidants, J. Chem. Soc., Perkin Trans. 2, (1998) 1289-1292.

[82] D.A. Becker, Highly Sensitive Colorimetric Detection and Facile Isolation of Diamagnetic Free Radical Adducts of Novel Chromotropic Nitrone Spin Trapping Agents Readily Derived from Guaiazulene, J. Am. Chem. Soc, 118 (1996) 905-906.

[83] J.S. Althaus, T.J. Fleck, D.A. Becker, E.D. Hall, P.F. Vonvoigtlander, Azulenyl Nitrones: Colorimetric Detection of Oxyradical End Products and Neuroprotection in the Gerbil Transient Forebrain Ischemia/Reperfusion Model, Free Rad. Biol. Med., 24 (1998) 738-744.

[84] P. Klivenyi, R.T. Matthews, M. Wermer, L. Yang, U. MacGarvey, D.A. Becker, R. Natero, M.F. Beal, Azulenyl Nitrone Spin Traps Protect against MPTP Neurotoxicity, Experimental Neurology, 152 (1998) 163-166.

[85] D.A. Becker, Diagnostic and therapeutic applications of azulenyl nitrone spin traps, Cell. Mol. Life Sci., 56 (1999) 626-633.

[86] D.A. Becker, J.J. Ley, L. Echegoyen, R. Alvarado, Stilbazulenyl Nitrone (STAZN): A Nitronyl-Substituted Hydrocarbon with the Potency of Classical Phenolic Chain-Breaking Antioxidants, J. Am. Chem. Soc, 124 (2002) 4678-4684.

[87] L. Yang, N.Y. Calingasan, J. Chen, J.J. Ley, D.A. Becker, M.F. Beal, A novel azulenyl nitrone antioxidant protects against MPTP and 3-nitropropionic acid neurotoxicities, Exp. Neurol., 191 (2005) 86-93.

[88] J.J. Ley, R. Prado, J.Q. Wei, N.H. Bishopric, D.A. Becker, M.D. Ginsberg, Neuroprotective antioxidant STAZN protects against myocardial ischemia/reperfusion injury, Biochem. Pharmacol., 75 (2008) 448-456.

[89] S.C. Mojumdar, D.A. Becker, G.A. DiLabio, J.J. Ley, L.R.C. Barclay, K.U. Ingold, Kinetic Studies on Stilbazulenyl-bis-nitrone (STAZN), a Nonphenolic Chain-Breaking Antioxidant in Solution, Micelles, and Lipid Membranes, J. Org. Chem., 69 (2004) 2929-2936.

[90] Lapchak, P.A., D.R. Schubert, P.A. Maher, De-Risking of Stilbazulenyl Nitrone (STAZN), a Lipophilic Nitrone to Treat Stroke Using a Unique Panel of In Vitro Assays, Translational Stroke Research, 2 (2011) 209-217.

[91] S.T. Caldwell, C. Quin, R. Edge, R.C. Hartley, A Dual Sensor Spin Trap for Use with EPR Spectroscopy, Organic Letters, 9 (2007) 3499-3502.

[92] L.E. Bakeeva, L.L. Grinius, A.A. Jasaitis, V.V. Kuliene, D.O. Levitsky, E.A. Liberman, I.I. Severina, V.P. Skulachev, Conversion of biomembrane-produced energy into electric form. II. Intact mitochondria, Biochim. Biophys. Acta, 216 (1970) 13-21.

[93] M.P. Murphy, R.A. Smith, Targeting antioxidants to mitochondria by conjugation to lipophilic cations, Annu. Rev. Pharmacol. Toxicol., 47 (2007) 629–656.

[94] R.A.J. Smith, C.M. Porteous, A.M. Gane, M.P. Murphy, Delivery of bioactive molecules to mitochondria in vivo, Proc. Natl. Acad. Sci. USA, 100 (2003) 5407-5412.

[95] R.A.J. Smith, C.M. Porteous, C.V. Coulter, M.P. Murphy, Selective targeting of an antioxidant to mitochondria, Eur. J. Biochem., 263 (1999) 709-716.

[96] M.P. Murphy, K.S. Echtay, F.H. Blaikie, J. Asin-Cayuela, H.M. Cocheme, K. Green, J.A. Buckingham, E.R. Taylor, F. Hurrell, G. Hughes, S. Miwa, C.E. Cooper, D.A. Svistunenko, R.A.J. Smith, M.D. Brand, Superoxide Activates Uncoupling Proteins by Generating Carbon-centered Radicals and Initiating Lipid Peroxidation: Studies using a Mitochondria-Targeted Spin Trap derived from alpha -phenyl-N-tert-butylnitrone, J. Biol. Chem., 278 (2003) 48534-48545.

[97] M. Hardy, F. Chalier, O. Ouari, J.-P. Finet, A. Rockenbauer, B. Kalyanaraman, P. Tordo, Mito-DEPMPO synthesized from a novel NH2-reactive DEPMPO spin trap: a new and improved trap for the detection of superoxide, Chem. Commun., 10 (2007) 1083-1085.

[98] M. Hardy, A. Rockenbauer, J. Vasquez-Vivar, C. Felix, M. Lopez, S. Srinivasan, N. Avadhani, P. Tordo, B. Kalyanaraman, Detection, characterization, and decay kinetics of ROS and thiyl adducts of mito-DEPMPO spin trap, Chem Res Toxicol, 20 (2007) 1053-1060.

[99] C. Quin, J. Trnka, A. Hay, M.P. Murphy, R.C. Hartley, Synthesis of a mitochondria-targeted spin trap using a novel Parham-type cyclization, Tetrahedron, 65 (2009) 8154-8160.

[100] L. Robertson, R.C. Hartley, Synthesis of N-arylpyridinium salts bearing a nitrone spin trap as potential mitochondria-targeted antioxidants, Tetrahedron, 65 (2009) 5284-5292.

[101] S. El Fangour, M. Marini, J. Good, S.J. McQuaker, P.G. Shiels, R.C. Hartley, Nitrones for understanding and ameliorating the oxidative stress associated with aging, Age, DOI 10.1007/s11357-009-9098-z (2009).

[102] A. Hay, M.J. Burkitt, C.M. Jones, R.C. Hartley, Development of a new EPR spin trap, DOD-8C (*N*-[4-dodecyloxy-2-(7'-carboxyhept-1'-yloxy)benzylidene]-*N*-*tert*-butylamine *N*-oxide), for the trapping of lipid radicals at a predetermined depth within biological membranes, Arch. Biochem. Biophys., 435 (2005) 336-346.

[103] A. Gamliel, M. Afri, A.A. Frimer, Determining radical penetration of lipid bilayers with new lipophilic spin traps, Free Rad. Biol. Med., 44 (2008) 1394-1405.

[104] E. Bodner, M. Afri, A.A. Frimer, Determining radical penetration into membranes using ESR splitting constants, Free Rad. Biol. Med., 49 (2010) 427-436.

[105] S. Morandat, G. Durand, A. Polidori, L. Desigaux, M. Bortolato, B. Roux, B. Pucci, PBN Derived Amphiphilic Spin-Traps. I. Synthesis and Study of Their Miscibility with Polyunsaturated Phospholipids, Langmuir, 19 (2003) 9699-9705.

[106] S. Ortial, S. Morandat, M. Bortolato, B. Roux, A. Polidori, B. Pucci, G. Durand, PBN derived amphiphilic spin-traps. II/Study of their antioxidant properties in biomimetic membranes, Coll. Surf. B, 113 (2014) 384– 393.

[107] Y. Han, Y. Liu, A. Rockenbauer, J.L. Zweier, G. Durand, F.A. Villamena, Lipophilic β-Cyclodextrin Cyclic−Nitrone Conjugate: Synthesis and Spin Trapping Studies, J. Org. Chem., 74 (2009) 5369-5380.

[108] M. Hardy, O. Ouari, L. Charles, J.-P. Finet, G. Iacazio, V. Monnier, A. Rockenbauer, P. Tordo, Synthesis and Spin-Trapping Behavior of 5-ChEPMPO, a Cholesteryl Ester Analogue of the Spin Trap DEPMPO, J. Org. Chem. , 70 (2005) 10426-10433.

[109] F. Choteau, G. Durand, I. Ranchon-Cole, C. Cercy, B. Pucci, Cholesterol-based a-phenyl-*N*-*tert*-butyl nitrone derivatives as antioxidants against light-induced retinal degeneration, Bioorg. Med. Chem. Lett., 20 (2010) 7405-7409.

[110] G. Durand, A. Polidori, J.P. Salles, M. Prost, P. Durand, B. Pucci, Synthesis and antioxidant efficiency of a new amphiphilic spin-trap derived from PBN and lipoic acid, Bioorg. Med. Chem. Lett., 13 (2003) 2673-2676.

[111] G. Durand, A. Polidori, O. Ouari, P. Tordo, V. Geromel, P. Rustin, B. Pucci, Synthesis and preliminary biological evaluations of ionic and nonionic amphiphilic alpha-phenyl-N-tert-butylnitrone derivatives, J. Med. Chem., 46 (2003) 5230-5237.

[112] G. Durand, B. Poeggeler, J. Böker, S. Raynal, A. Polidori, M.A. Pappolla, R. Hardeland, B. Pucci, Fine-tuning the amphiphilicity: A crucial parameter in the design of potent alpha-phenyl-N-tert-butylnitrone analogues, J. Med. Chem. , 50 (2007) 3976-3979.

[113] T. Asanuma, H. Yasui, O. Inanami, K. Waki, M. Takahashi, D. Iizuka, T. Uemura, G. Durand, A. Polidori, Y. Kon, B. Pucci, M. Kuwabara, A new amphiphilic derivative, *N*-{[4-(lactobionamido)methyl]benzylidene}-1,1-dimethyl-2-(octylsulfanyl)ethylamine *N*-oxide, has a protective effect against copper-induced fulminant hepatitis in *Long-Evans* cinnamon rats at an extremely low concentration compared with its original form a-phenyl-*N*-(*tert*-butyl) nitrone, Chemistry & Biodiversity, 4 (2007) 2253-2267.

[114] G. Durand, A. Polidori, J.-P. Salles, B. Pucci, Synthesis of a new family of glycolipidic nitrones as potential antioxidant drugs for neurodegenerative disorders, Bioorg. Med. Chem. Lett., 13 (2003) 859-862.

[115] B. Poeggeler, G. Durand, A. Polidori, M.A. Pappolla, I. Vega-Naredo, A. Coto-Montes, J. Boeker, R. Hardeland, B. Pucci, Mitochondrial medicine: Neuroprotection and life extension by the new amphiphilic nitrone LPBNAH acting as a highly potent antioxidant agent, J. Neurochem., 95 (2005) 962-973.

[116] S. Tanguy, G. Durand, C. Reboul, A. Polidori, B. Pucci, M. Dauzat, P. Obert, Protection against reactive oxygen species injuries in rat isolated perfused hearts: Effect of LPBNAH, a new amphiphilic spin-trap derived from PBN, Cardiovascular Drugs and Therapy, 20 (2006) 147-149.

[117] G. Durand, B. Poeggeler, S. Ortial, A. Polidori, F.A. Villamena, J. Böker, R. Hardeland, M.A. Pappolla, B. Pucci, Amphiphilic amide nitrones: A new class of protective agents acting as modifiers of mitochondrial metabolism, J. Med. Chem., 53 (2010) 4849-4861.

[118] E. Chabaud, P. Barthelemy, N. Mora, J.L. Popot, B. Pucci, Stabilization of integral membrane proteins in aqueous solution using fluorinated surfactants, Biochimie, 80 (1998) 515-530.

[119] S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M.A. Pappolla, J. Boeker, R. Hardeland, B. Pucci, Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents, J. Med. Chem., 49 (2006) 2812-2820.

[120] G. Durand, R.A. Prosak, Y. Han, S. Ortial, A. Rockenbauer, B. Pucci, F.A. Villamena, Spin trapping and cytoprotective properties of fluorinated amphiphilic carrier conjugates of cyclic versus linear nitrones, Chem. Res. Toxicol., 22 (2009) 1570-1581.

Chapter II

Towards the improvement of the intrinsic properties of α-Phenyl-*N*-*tert*-butyl Nitrone

Chapter II – Part 1

Reactivities of Substituted α-Phenyl-*N*-*tert*-butyl Nitrones

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I. Introduction

The addition of free radicals to nitrones yields a persistent aminoxyl-based spin-adduct that can be detected and characterized by electron paramagnetic resonance (EPR) spectroscopy. Spin trapping by EPR spectroscopy is a popular method for the detection of free radicals in chemical and biological systems.[1] The α-phenyl-*N*-*tert*-butylnitrone (PBN) and its derivatives are widely employed as spin-traps in *in-vitro*, *in-vivo* and *ex-vivo* systems.[2, 3] Aside from their application as spin-traps, nitrones have also exhibited a variety of protective properties in animal models against oxidative stress mediated injury.[4, 5] However, despite the promising pharmacological properties of PBN, the molecular mechanism of its action is not well understood. Of the many PBN derivatives that have been synthesized over the years, disodium-[(tert-butylimino)-methyl]benzene-1,3-disulfonate N-oxide (NXY-059) has gained the most attention since it is the first neuroprotective agent that had reached the phase 3 clinical trial in the USA.[6] Although it has been suggested that the radical trapping properties of NXY-059 is the basis of its neuroprotective action, experimental evidences suggest other possible mechanisms being involved.

One of the promising strategies in the design of novel nitrone-based spin-traps is to selectively target these compounds in relevant sites of radical production, mainly the mitochondrial electron transport chain, the cytosol, and the membrane bound NAD(P)H oxidase.[7-9] Selective targeting is usually achieved by conjugating the nitronyl group to specific target ligands, and therefore, the choice of linker groups for optimal spin tapping properties is highly desirable. In addition to the type of ligands that are tethered to nitrones, it has been demonstrated that the nature of the linker group also affects its bioactivity.[10]

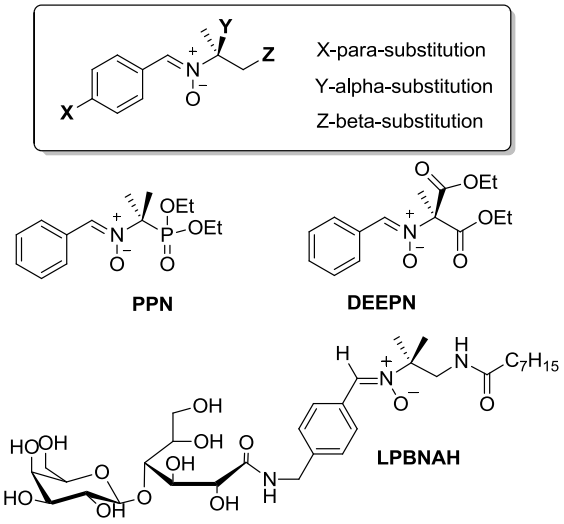


Figure 2.1.1. General structure and some examples of substituted linear nitrones.

Only a relatively limited number of *N*-*tert*-butyl substituted nitrones have been synthesized over the past years where functionalization of the aromatic ring being the most facile. Several *N*-*tert*-butyl substituted PBNs have been synthesized such as *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN), [11] *N*-benzylidene-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPN),[12] or the amide amphiphilic nitrones developed by our group (LPBNAH),[9] (Figure 2.1.1), but the effect of the substituents on the electronic properties of the nitrones and their reactivity to radicals such as $O_2^{\bullet-}$ are not known. The substituent effect on the reactivity of DMPO-type cyclic nitrones has been extensively studied through experimental and computational approaches[13-15] demonstrating the nucleophilic nature of $O_2^{\bullet-}$ addition to C-5-substituted DMPO nitrones.[13]. Therefore, derivatization of the *tert*-butyl group of the PBN may exhibit electronic properties that enhance $O_2^{\bullet-}$ addition to nitrones and offers opportunities for multi-functionalization of the spin-trap for subcellular target specificity and controlled delivery in *in-vitro* and *in-vivo* systems.

Our previous computational and kinetic studies showed that para-substitution by electron withdrawing substituents in PBN gave no significant polar effects on their reactivity towards

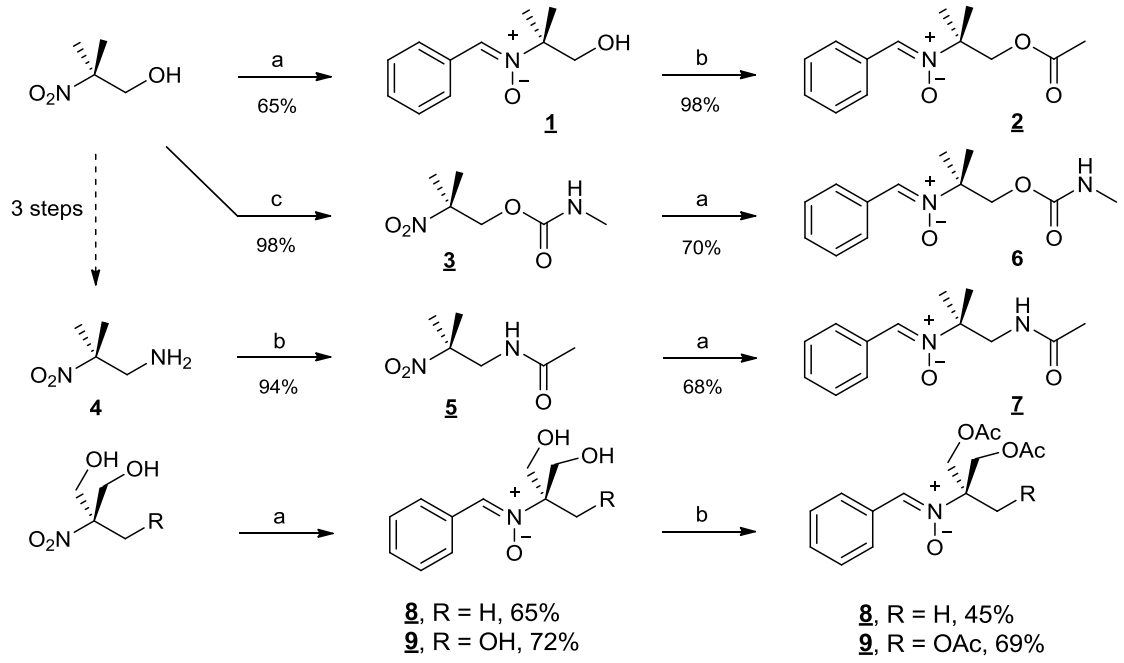
$O_2^{\bullet-}$, while with $HO_2^{\bullet}$, a more pronounced increase in the kinetic of addition was observed indicating the electrophilic nature of this reaction. Our current goal is to explore the reactivity of various *N*-*tert*-butyl substituted phenyl nitrones so as to identify the most optimal linker groups. Through optimization of the linkers, we would be able to design selective targeted nitrone-based spin-traps with improved reactivity towards free radicals.

In this work, five new *N*-*tert*-butyl substituted PBNs were synthesized along with a series of mono-, di- and tri-hydroxymethyl *N*-*tert*-butyl substituted PBN derivatives. The water solubility, lipophilicity and electrochemical properties were determined. A UV-Vis stopped-flow competitive technique was employed to determine the relative rate constants of reaction with $O_2^{\bullet-}$. The relative rate constants of phenyl adduct formation were experimentally determined by EPR competition kinetic technique. Using a computational approach, the effect of the *N*-*tert*-butyl substituents on the nitronyl-atom charge density and electron density localization as well as on the free energies of nitrone reactivity with $O_2^{\bullet-}$and $HO_2^{\bullet}$ were calculated. Finally, the cytroprotective property of selected compounds against oxidant-induced cell death was investigated.

II. Results and Discussion.

Synthesis of *tert*-substituted nitrones. All the mono-, di- and trisubstituted nitrones with the substituents in β-position (See Figure 2.1.1 for details) were synthesized by a one pot reduction/condensation of nitro derivatives onto the commercially available benzaldehyde as shown in Scheme 1. The PBN-CH_2OH[16] was synthesized from 2-methyl-2-nitro-1-propanol and benzaldehyde in the presence of zinc powder and AcOH in ethanol according to our recently described procedure.[17] After purification by flash chromatography and two successive crystallizations from EtOAc/*n*-hexane, nitrone **1** was obtained in 65% yield which is slightly higher than the procedure used by Janzen and Zawalsky (55%).[16] Acetylation of

nitrone **1** by a mixture of Ac_2O/pyridine 1:1 v/v led to compound **2** (also called PBN-CH_2OAc) in 98% yield, after purification by flash chromatography. The synthesis of compound **3** was carried out in one step. First, 2-methyl-2-nitro-1-propanol was activated using 1,1'-carbonyldiimidazole (CDI) in the presence of 4-dimethylaminopyridine (DMAP) in THF then methylamine was added to the reaction mixture to give after purification compound **3** in 98% yield. In parallel, 2-methyl-2-nitro propanamine **4** was obtained from 2-methyl-2-nitro-1-propanol in three steps[9] and was then acetylated to give the nitro compound **5** in 94% yield. The one pot reduction/condensation of compounds **3** and **5** to benzaldehyde after purification by flash chromatography and two successive crystallization from EtOAc/*n*-hexane led to nitrones **6** (also called PBN-$CH_2OCONHMe$) and **7** (also called PBN-CH_2NHAc) in 70% and 68% yield, respectively.



Scheme 2.1.1. Synthesis of mono-, di- and tri-*β*-substituted nitrones. Reagents and conditions: (a) Benzaldehyde, zinc powder, AcOH, 4 Å molecular sieves; ethanol, 15→ 60°C, 10 h; (b) Ac_2O/pyr (1:1 v/v), rt, 12h; (c) CDI, DMAP, THF, 2h, rt, then CH_3NH_2, 18h, rt.

Following the same synthetic procedure, the PBN-$(CH_2OH)_2$ **8** was also synthesized from 2-methyl-2-nitro-1,3-propadinol in 65% yield, which corresponds to a significant improvement compared to the procedure used by Janzen and Zawalsky with15%.[16] The PBN-$(CH_2OH)_3$ **9** was obtained following our recent procedure from 2-hydroxymethyl-2-nitro-1,3-propanediol.[17] Finally, PBN-$(CH_2OAc)_2$ **10** and PBN-$(CH_2OAc)_3$ **11** were obtained by acetylation of nitrone **8** and **9**, respectively.

Water solubility & partition coefficient. Nitrones **1**, **7** and **8** are soluble in water up to a concentration of ~ 200 g/L, after which the solution becomes viscous but remained transparent. This is significantly higher than PBN whose solubility limit was found to be ~ 21 g/L. When comparing the amide and carbamate derivatives, **7** and **6**, a significant difference was noted with **6** exhibiting a water-solubility limit of ~ 11 g/L. Carbamates are indeed known to be hardly soluble in water.[18] We also demonstrated that the solubility of hydroxylated compounds was not linearly correlated with the number of hydroxyl groups. Whereas, the mono- and di-substituted hydroxyl compounds are highly soluble in water (>200 g/L), the tri-substituted one reaches its solubility limit at ~ 21 g/L likely due intra-molecular hydrogen bonding between the three hydroxyl groups as previously observed.[17] Due to the oily form of the three ester derivatives their solubility was not determined.

The relative lipophilicity (log k'_w) of the nitrones was measured by HPLC and values are reported in Table 1. This confirms the higher lipophilic character of the three ester compounds compared to PBN with log k'_w values of 1.89, 1.95 and 2.17 for compounds **2**, **10** and **11**, respectively, whereas 1.64 was found for PBN. Although compounds **6** and **7** exhibit different water-solubility, they were both found to have similar lipophilicity, slightly lower than that of PBN. Finally, the hydroxylated derivatives **1**, **8** and **9** were found to be the least lipophilic derivatives where the lipophilicity correlates with the number of hydroxyl groups, that is, the lower the number of hydroxyl groups, the higher the lipophilicity. Calculated partition

coefficients (Clog*P*) were also determined using Marvin software. Except for the three ester derivatives, a good correlation between log *k*'w and Clog*P* was obtained (Figure 2.1.2).

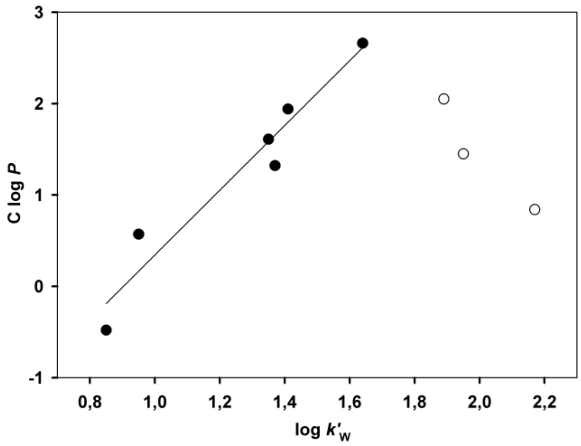


Figure 2.1.2. Correlation between log *k*'w and Clog*P*. Hydroxylated compounds **1**, **8** and **9** are marked as (○).

Table 2.1.1. Physical-chemical and electrochemical properties of PBN derivatives.

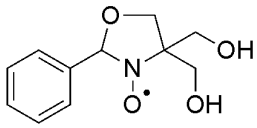
Compounds		**Water solubility (g/L)**	**Lipophilicity**		E_p**(c) (V)**			E_p**(a) (V)**
			log *k*'w[b]	Clog *P*[c]	In H_2O[d]	In CH_3CN[e]		In CH_3CN[e]
						2nd Peak	1st Peak	
PBN-CH_2OH	**1**	>200	1.35	1.61	-1.70	-2.12	-1.92	1.57
PBN-CH_2OAc	**2**	nd[a]	1.89	2.05	-1.69	-2.40	-2.03	1.67
PBN-$CH_2OCONHMe$	**6**	10.8	1.41	1.94	-1.71	-2.14	-1.96	1.77
PBN-CH_2NHAc	**7**	>200	1.37	1.32	-1.70	-2.15	-1.97	1.44
PBN-$(CH_2OH)_2$	**8**	>200	0.95	0.57	-1.74	-2.29	-2.12	1.55
PBN-$(CH_2OH)_3$	**9**	21.4	0.85	-0.48	-1.67	-2.27	-2.08	1.58
PBN-$(CH_2OAc)_2$	**10**	nd[a]	1.95	1.45	-1.72	-2.31	-1.93	1.76
PBN-$(CH_2OAc)_3$	**11**	nd[a]	2.17	0.84	-1.75	-2.28	-1.89	1.83
PBN		21.4	1.64	2.66	-1.70	-2.23	-2.10	1.60

[a]Not determined. [b]Partition coefficient values obtained by HPLC. [c]Calculated octanol/water partition coefficient values obtained using Marvin software (http://www.chemaxon.com/marvin/help/index.html). [d]Containing 50 mM of NaCl. [e]Containing 50 mM of TBAP.

Table 2.1.2. EPR hyperfine coupling constant of different radical adducts of β-substituted nitrones.

Radical adducts			PBN-CH_2OH (**1**)			PBN-CH_2OAc (**2**)		PBN-$CH_2OCONHMe$ (**6**)		PBN-CH_2NHAc (**7**)			PBN-$(CH_2OH)_2$ (**8**)			PBN-$(CH_2OH)_3$ (**9**)		
	Source	Solvent	a_N	a_H	ratio	a_N	a_H	a_N	$a_{b\text{-}H}$	a_N	a_H	ratio	a_N	a_H	ratio	a_N	a_H	ratio
$HO^{\bullet}$	Fenton	PBS	15.5	2.5	2:3	15.2	2.8	15.2	2.9	15.1	2.9	-	15.2	2.4	1:3	15.3	19.7	
			15.9	20.1	1:3								15.6	19.9	2:3			
$O_2^{\bullet-}$	KO_2	DMSO	14.9	17.9	2:3	14.0	1.6	14.9	0.4	14.6	16.5	1:2	14.7	2.8	2:3	14.5	2.3	3:4
			14.2	-	1:3					13.6	2.4	1:2	14.9	18.1	1:3	14.6	18.5	1:4
$HOO^{\bullet}$	H_2O_2	pyridine	13.3	1.5	-	13.1	1.4	13.3	1.6	13.3	1.7	-	13.1	1.2	-	13.1	1.1	3:4
																14.1	2.0	1:4
$CH_3O^{\bullet}$	MeOH $Pb(OAc)_4$	DMSO	15.0	18.0	4:5	13.5	2.4	13.6	2.5	13.7	2.4	-	13.1	0.35	1:3	12.9	2.1	-
			13.4	0.8	1:5								12.8	1.9	2:3			
$C_6H_5^{\bullet}$	C_6H_5-I/UV	benzene	14.5	2.5	-	13.6	2.2	14.4	3.0	14.1	2.3	-	14.2	2.3		14.1	2.0	

Spin trapping. To evaluate the spin trapping ability of the PBN substituted derivatives, we investigated the formation of various oxygen-centered radical spin-adducts *i.e*. $HO^{\bullet}$, $O_2^{\bullet-}$, and $MeO^{\bullet}$ adducts. The hyperfine coupling constants (hfcc's) of the mono substituted derivatives **1, 2, 6-7** and those of the di- and tri-hydroxylated derivatives **8-9** are reported in Table 2.1.2. In most cases, the nitrones tested gave rise to a standard six-line EPR spectrum whose values are in agreement with the literature.[19] Two different conditions were used to generate the superoxide adducts that is pyridine/H_2O_2 and DMSO/KO_2. In the pyridine/H_2O_2 system, one predominant radical adduct was detected in most cases with hfcc values in agreement with a $O_2^{\bullet-}$ adduct. In the KO_2 system, the hyperfine coupling constants suggest $O_2^{\bullet-}$ adduct formation with higher values than in pyridine/H_2O_2 system which is likely due to solvent effect as previously observed for para-substitued nitrones.[20] For the hydroxylated derivatives, the presence of a second nitroxide having a six-line pattern spectrum was also observed. This second species corresponds to an oxazolidine-*N*-oxyl compound coming from a cyclization reaction between one hydroxyl group and the nitronyl carbon.[17]

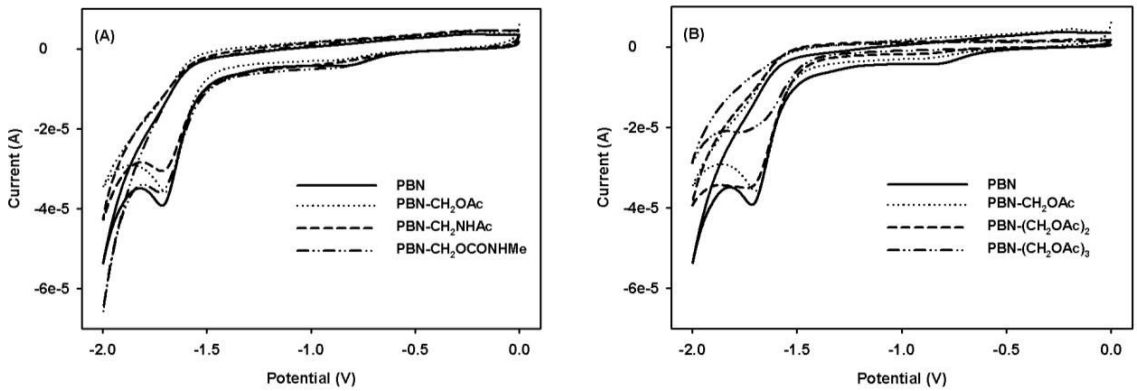


Under Fenton condition, the ratio of the cyclic species increased with the number of hydroxyl groups: ~ 1/3 for compound **1** (a_N = 15.9, a_H = 20.1), ~ 2/3 for compound **8** (a_N = 15.6, a_H = 19.9) while for compound **9** bearing three hydroxyl groups (a_N = 15.3, a_H = 19.7), the cyclic species was predominant (>90%). This suggests that cyclization may be favored with an increasing number of hydroxyl groups and a thorough investigation on this is currently in progress in our labs. The formation of five-membered cyclic nitroxides was also evident in KO_2 system for the hydroxylated derivatives, however, in this case, the ratio of the cyclic nitroxide decreased with the number of hydroxyl groups. Cyclisation was observed during methoxy radical trapping for the monohydroxylated compound but not for the di-and tri-

hydroxylated derivatives. Regardless of the radical generating system used, no evidence of cyclization was found for the carbamate- and ester-based compounds, while for the amide derivative, cyclisation was only evident in the KO_2 system. This suggests that cyclization involving a nitrogen atom may also occur in basic condition.

We also investigated the trapping of a carbon-centered radical. The phenyl radical spin-adducts was obtained by photolysis of a phenyliodide solution in benzene in the presence of the nitrones. Although, all the nitrones tested trapped $Ph^{\bullet}$ giving rise to a standard six-line EPR spectrum, it should be noted that a weak signal was obtained with the trihydroxylated compound **9**. In all cases, a_N and a_H values determined are in agreement with an aryl radical adduct of a PBN-type nitrone.

Cyclic Voltammetry. The oxidative and reductive character of these nitrones was investigated using cyclic voltammetry and values are reported in Table 1. We first carried out cyclic voltammetry in 50 mM NaCl aqueous solution. As already observed for other nitrones, the oxidation of the nitronyl group was not detected.[21, 22] On the contrary, we observed that all the nitrones exhibited an irreversible one-step reduction, as shown in Figure 2.1.3.



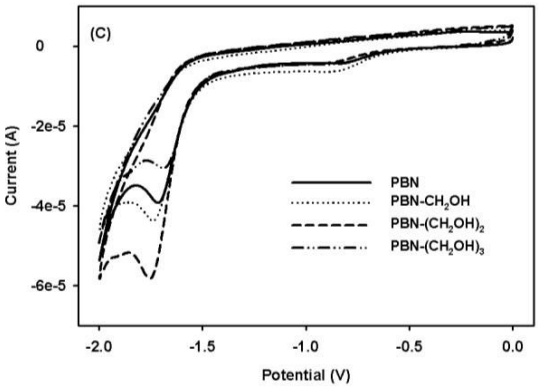


Figure 2.1.3. Cyclic voltammograms of PBN and compounds **2**, **6** and **7** (A); PBN and compounds **2**, **10** and **11** (B); PBN and compounds **1**, **8** and **9** (C). Reduction in water containing 50 mM of NaCl with a sweep rate of 0.1 $V.s^{-1}$.

The cathodic peak potential of the β-substituted derivatives is observed between -1.67 V and -1.75 V vs. Ag/AgCl, and that of the PBN being at -1.70 V. This is consistent with the findings by Zuman and Exner[23] who reported the weak influence of *N*-alkyl substituents on the reduction potential of α-phenyl-*N*-alkylnitrones, which was further confirmed by McIntire et al.[21] We next studied the electrochemical properties of the nitrones in acetonitrile containing tetra-butylammonium perchlorate (TBAP) as electrolyte. Previous works showed that PBN undergoes an irreversible one-electron oxidation and a one-step, two-electron reduction.[21, 24, 25] Compared to the aqueous conditions, oxidation of nitrones was clearly observed in acetonitrile as shown in Figure 2.1.4 and Figure 2.1.5 with values ranging from 1.44 V to 1.83 V. For the monosubstituted derivatives, the highest observed oxidation potential was for the carbamate derivative **6** followed by the ester **2** and then by the hydroxylated **1** which suggest a strong inductive effect of the carbamate bond, making nitrone **6** harder to oxidize than nitrones **2** and **1**. This shows that the presence of β-substituents affects the oxidation of the nitronyl function. The amide compound **7** with the lowest anodic peak potential in the series is, therefore, the easiest to oxidize demonstrating that the oxidation of the nitronyl group is more difficult in the presence of electron-withdrawing substituents, in agreement with the literature.[21, 25] With regard to the number of substituents, the oxidation

potential of the ester derivatives **2**, **10** and **11** increases with the number of substituents suggesting that the electronic effects are additive. No significant trend was observed for the mono-, di- and tri-hydroxylated derivatives whose potential was close to that of PBN, in agreement with the literature.[21]

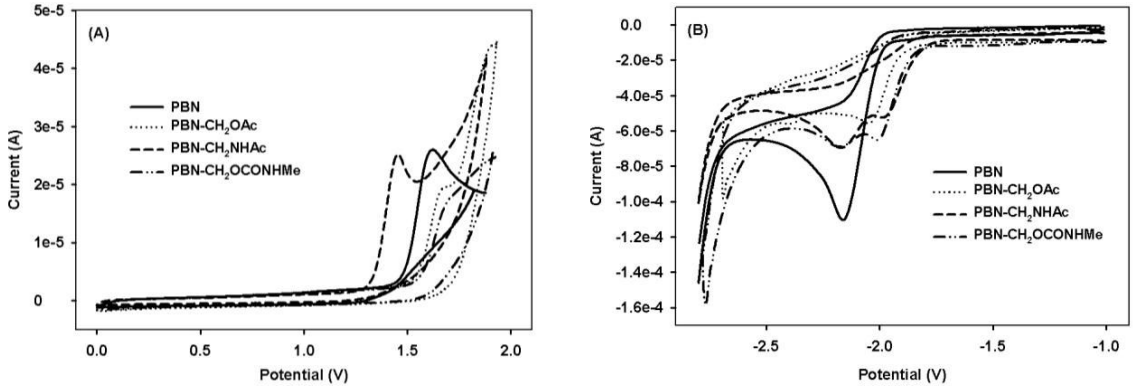


Figure 2.1.4. Cyclic voltammograms of PBN and compounds **2**, **6** and **7** in acetonitrile containing 50 mM of TBAP with a sweep rate of 0.1 $V.s^{-1}$; (A) oxidation and (B) reduction.

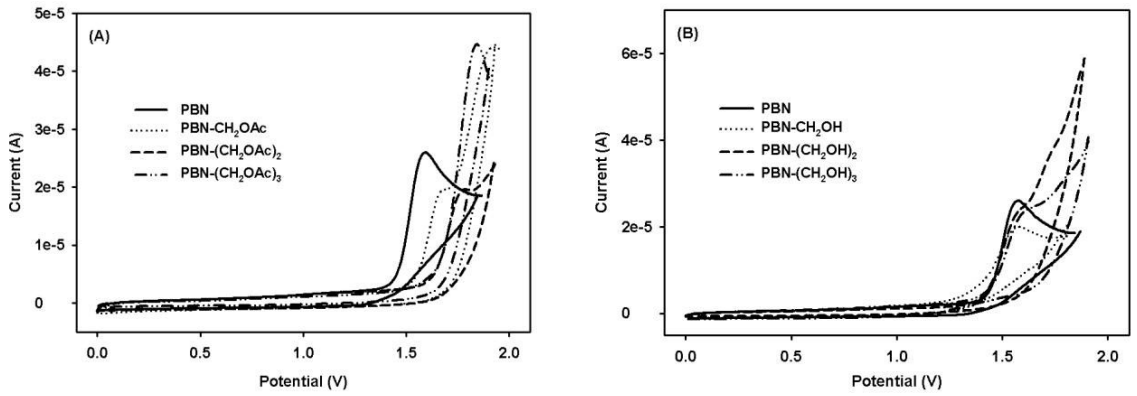


Figure 2.1.5. Cyclic voltammograms of PBN and compounds **2**, **10** and **11**(A); PBN and compounds **1**, **8** and **9** (B). Oxidation in acetonitrile containing 50 mM of TBAP with a sweep rate of 0.1 $mV.s^{-1}$.

The reduction of nitrones in a non-aqueous medium was then investigated and exhibited two reduction potentials for all the β-substituted derivatives, whereas for PBN, only one reduction peak was observed (Figure 2.1.4 and Figure 2.1.6).

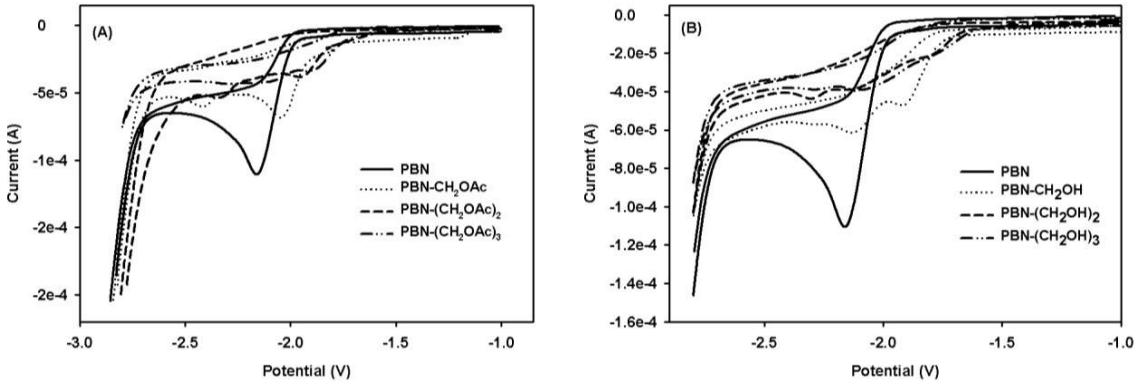


Figure 2.1.6. Cyclic voltammograms of PBN and compounds **2**, **10** and **11** (A); PBN and compounds **1**, **8** and **9** (B). Reduction in acetonitrile containing 50 mM of TBAP with a sweep rate of 0.1 $V.s^{-1}$.

The presence of two reduction potentials had been observed for β-phosphorylated nitrone spin-traps.[24] For the monosubstituted derivatives, only a modest ease of reduction was observed compared to PBN with only ~ 0.1-0.2 V shift in potential. The reduction of the ester derivatives becomes slightly easier with increasing number of substituent suggesting an additive effect as also observed for the oxidation which is contrary to the hydroxylated derivatives where no correlation was found. We further studied the influence of the sweep rate of PBN and compounds **2** and **7** on the anodic and cathodic peak current densities. The plot of current intensity versus square root of the sweep rate showed a linear decrease for the reduction and a linear increase for the oxidation, as shown in Figures 2.1.7 and 2.1.8, demonstrating that the electrochemical process is diffusion-controlled.[26, 27] The diffusion coefficients of the three nitrones were found in the same range for both oxidation and reduction. The reduction of nitrones **2** and **7** exhibited two separate reduction potentials where the current intensity and the square scan speed were linear for both peaks.

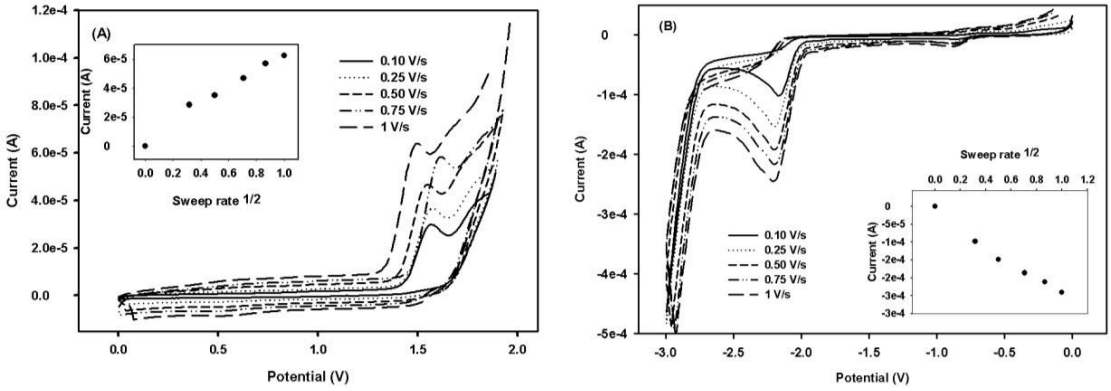


Figure 2.1.7. Sweep rate variations on PBN oxidation (A) and reduction (B) in acetonitrile containing 50 mM of TBAP at sweep rate ranging from 0.1 $V.s^{-1}$ to 1 $V.s^{-1}$ with corresponding linear regression curve.

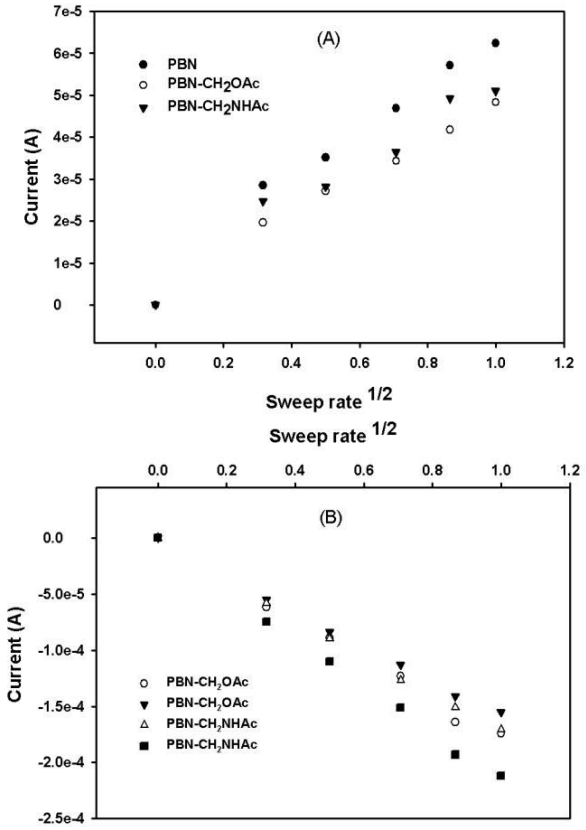


Figure 2.1.8. Linear regressions corresponding to sweep rate variations on PBN, PBN-CH_2OAc and PBN-CH_2NHAc oxidation (A) and reduction (B) in acetonitrile containing 50 mM of TBAP at sweep rate ranging from 0.1 $V.s^{-1}$ to 1 $V.s^{-1}$.

Nitronyl atoms charge density correlation with NMR chemical shift. Examination of the optimized structures at the B3LYP/6-31G* level of theory shows C=N and N-O bond distances that are in the range of 1.312-1.317 Å and 1.276-1.292 Å, respectively, which are consistent with the X-ray crystallographic C=N and N-O bond lengths observed for *N*-tert-butyl-α-(2-pyridyl)nitrone of 1.307 Å and 1.294 Å, respectively.[28] The natural population analysis (NPA) charges on the nitronyl-carbon, nitronyl-nitrogen, and nitronyl-oxygen atoms were determined at the PCM/B3LYP/6-31+G** level for compounds **1-3** and **5-11** as well as for the other *N-tert*-butyl substituted derivatives (Table 2.1.3).

Table 2.1.3. Natural population analysis (NPA) charge densities of the nitronyl- atoms of PBN derivatives at the PCM/B3LYP/6-31+G**//B3LYP/6-31G* level of theory. In bold are the data for the molecules synthesized.

Nitrones		monosubstitution			disubstitution			trisubstitution		
		C	**N**	**O**	**C**	**N**	**O**	**C**	**N**	**O**
PBN	$-CH_3$	**0.009**	**0.073**	**-0.606**	n/a	n/a	n/a	n/a	n/a	n/a
α-substitution	-COOH	0.029	0.067	-0.607	0.041	0.059	-0.606	0.050	0.056	-0.590
	-C(=O)OMe	0.005	0.077	-0.577	0.037	0.063	-0.601	0.040	0.061	-0.576
	$-C(=O)NH_2$	0.043	0.055	-0.617	0.074	0.037	-0.631	0.068	0.039	-0.613
	-OMe	0.014	0.047	-0.595	0.020	0.030	-0.601	0.023	0.016	-0.595
	-OC(=O)Me	0.028	0.050	-0.596	0.034	0.028	-0.592	0.040	0.012	-0.577
	-OC(=O)NHMe	0.016	0.049	-0.601	0.049	0.017	-0.617	0.049	0.018	-0.584
	-NHC(=O)Me	0.023	0.057	-0.609	0.005	0.055	-0.583	0.004	0.046	-0.564
	-SMe	0.017	0.057	-0.596	0.012	0.048	-0.584	0.014	0.035	-0.567
	$-P(=O)(OMe)_2$	0.021	0.048	-0.586	0.031	0.047	-0.575	0.051	0.028	-0.582
β-substitution	$-CH_2OH$	**0.015**	**0.070**	**-0.608**	**0.015**	**0.074**	**-0.605**	**0.023**	**0.062**	**-0.613**
	$-CH_2OC(=O)Me$	**0.023**	**0.065**	**-0.608**	**0.025**	**0.062**	**-0.605**	**0.037**	**0.053**	**-0.608**
	$-CH_2NHC(=O)Me$	**0.028**	**0.063**	**-0.619**	0.035	0.059	-0.620	0.052	0.049	-0.624
	$-CH_2OC(=O)NHMe$	**0.023**	**0.066**	**-0.608**	0.041	0.150	-0.241	0.027	0.056	-0.596
	$-CH_2C(=O)NH_2$	0.036	0.054	-0.621	0.061	0.042	-0.633	0.038	0.050	-0.609
	$-CH_2C(=O)OMe$	0.023	0.067	-0.610	0.034	0.061	-0.611	0.034	0.063	-0.604
	$-CH_2OMe$	0.016	0.070	-0.607	0.020	0.067	-0.609	0.022	0.066	-0.610
	$-CH_2P(=O)(OMe)_2$	0.030	0.054	-0.609	0.024	0.055	-0.601	0.008	0.068	-0.566
	$-CH_2SMe$	0.016	0.070	-0.604	0.018	0.071	-0.597	0.022	0.067	-0.594

In general, increasing the number of substitution results in more positive charge densities on the nitronyl-C with the exception of few compounds such as PBN-NHC(=O)Me and PBN-$CH_2P(=O)(OMe)_2$, whereas for the nitronyl-N, an opposite but less pronounced trend was observed. As for the nitronyl-O, no significant effect of the substitution was observed throughout the series of *N-tert*-butyl substituted derivatives. This observation is consistent with increased distribution of the mesomeric B to the resonance hybrid form in the presence

of multiple substituents, where there is an increased electron density on the nitronyl-N and a decreased electron density on the nitronyl-C. This is further supported by natural bond orbital (NBO) analysis showing that there is a decrease in the percent (0.25%-2.97%) of electron localization on the nitronyl-C with increasing substitution (from mono to tri) except for PBN–NHC(O)Me and PBN-$CH_2P(=O)(OMe)_2$ where there is an increase in electron distribution (Table 2.1.4).

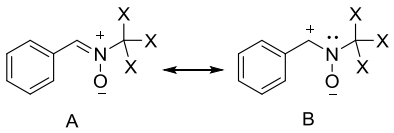


Table 2.1.4. Natural bond orbital (NBO) analysis showing the percent electron localization on the polar σ C-N and N-O (in parentheses); and π C-N bonds at the B3LYP/6-31+G** level of theory.

Nitrones		monosubstitution			disubstitution			trisubstitution		
		C	**N**	**O**	**C**	**N**	**O**	**C**	**N**	**O**
PBN	-CH_3 σ	37.07	62.93 (50.13)	49.87	n/a	n/a	n/a	n/a	n/a	n/a
α-substitution	π	40.32	59.68		n/a	n/a	n/a	n/a	n/a	n/a
	-COOH σ	36.87	63.13 (50.45)	49.55	36.49	63.51 (50.62)	49.38	36.96	63.61 (50.78)	49.22
	π	39.16	60.84		37.12	62.88		36.19	63.81	
	-C(=O)OMe σ	37.02	62.98 (50.17)	49.83	36.54	63.46 (50.54)	49.46	36.52	63.48 (50.67)	49.33
	π	39.85	60.15		37.45	62.55		36.98	63.02	
	-$C(=O)NH_2$ σ	37.01	62.99 (50.16)	49.84	36.93	63.07 (50.19)	49.81	36.71	63.29 (50.61)	49.39
	π	38.41	61.59		36.72	63.28		36.21	63.79	
	-OMe σ	37.19	62.81 (50.12)	49.88	36.97	63.03 (50.32)	49.68	37.05	62.95 (50.27)	49.73
	π	39.91	60.09		38.36	61.64		37.93	62.07	
	-OC(=O)Me σ	36.97	63.03 (50.33)	49.67	36.83	63.17 (50.41)	49.59	36.76	63.24 (50.89)	49.11
	π	38.96	61.04		37.45	62.55		36.79	63.21	
	-OC(=O)NHMe σ	36.93	63.07 (50.39)	49.61	36.91	63.09 (50.19)	49.81	37.10	62.90 (50.14)	49.86
	π	39.29	60.71		36.62	63.38		36.92	63.08	
	-NHC(=O)Me σ	37.06	62.94 (50.13)	49.87	37.19	62.18 (49.95)	50.05	37.39	62.61 (49.62)	50.38
	π	39.13	60.87		40.01	59.99		39.73	60.27	
	-SMe σ	36.93	63.07 (50.24)	49.76	36.77	63.23 (50.39	49.61	36.79	63.21 (50.26)	49.74
	π	39.52	60.48		39.08	60.92		38.21	61.79	
	-$P(=O)(OMe)_2$ σ	36.90	63.10 (50.38)	49.62	36.59	63.41 (50.44)	49.56	36.38	63.62 (50.49)	49.51
	π	38.30	61.70		37.84	62.16		36.09	63.91	
β-substitution	-CH_2OH σ	37.01	62.99 (50.22)	49.78	36.99	63.01 (50.22)	49.78	36.76	63.24 (50.33)	49.67
	π	39.88	60.12		40.12	59.88		37.95	62.05	
	-$CH_2OC(=O)Me$ σ	36.96	63.04 (50.29)	49.71	36.85	63.15 (50.29)	49.71	36.73	63.27 (50.49)	49.51
	π	39.43	60.57		38.38	61.62		37.34	62.66	
	-$CH_2NHC(=O)Me$ σ	37.06	62.94 (50.08)	49.92	36.98	63.02 (50.16)	49.84	36.96	63.04 (50.16)	49.84
	π	39.41	60.59		38.68	61.32		38.08	61.92	
	-$CH_2OC(=O)NHMe$ σ	36.96	63.04 (50.29)	49.71	36.86	63.14 (50.39)	49.61	36.82	63.18 (50.46)	49.54
	π	39.46	60.54		38.43	61.57		37.82	62.18	
	-$CH_2C(=O)NH_2$ σ	37.07	62.93 (50.15)	49.85	37.12	62.88 (49.93)	50.07	37.00	63.00 (50.26)	49.74
	π	38.82	61.18		37.68	62.32		38.14	61.86	
	-$CH_2C(=O)OMe$ σ	36.97	63.03 (50.22)	49.78	36.94	63.06 (50.24)	49.76	36.75	63.25 (50.19)	49.81
	π	39.29	60.71		38.79	61.21		38.70	61.30	
	-CH_2OMe σ	37.01	62.99 (50.23)	49.77	36.93	63.07 (50.31)	49.69	36.81	63.19 (50.34)	49.66
	π	39.92	60.08		39.54	60.46		38.65	61.35	
	-$CH_2P(=O)(OMe)_2$ σ	36.97	63.03 (50.27)	49.73	37.03	62.97 (50.16)	49.84	37.06	62.94 (49.71)	50.29

The effect of the nature of substituent on the charge density of the nitrone moiety was also studied using ^{1}H and ^{13}C NMR spectroscopy. Similar to carbonyl compounds, nitrones are susceptible to nucleophilic addition reaction, and therefore, the electronic nature of the nitronyl-C can affect its reactivity towards nucleophilic radicals such as $O_2^{\bullet -}$. Figure 2.1.10.A shows good correlation between the ^{13}C NMR chemical shift in $CDCl_3$ of the nitronyl-C and the calculated nitronyl-C charge density where there is a downfield shift with increasing positive charge of the nitronyl-C. This confirms the presence of a polar effect from the substituent in β position on the nitronyl charge density and suggests a stabilization of the mesomeric B form due to the electron withdrawing effect of the substituents. Only the mono- and dihydroxylated derivatives **1** and **8** looked out of the range, likely due to the formation of intra-molecular hydrogen bonding between the hydroxyl group and the nitronyl-O,[17] which may induce a downfield shift. An opposite trend was observed for the ^{1}H NMR chemical shift in $CDCl_3$ of the nitronyl-H where an upfield shift of the β-hydrogen was observed with increased positivity of the nitronyl-C further confirming the polar effect from the *N-tert*-butyl substituents. (Figure 2.1.9).

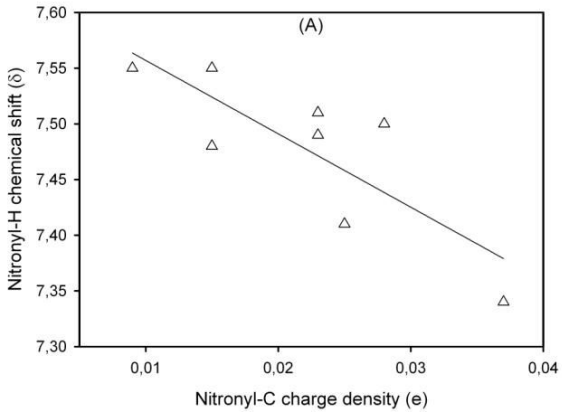


Figure 2.1.9. Correlation of the nitronyl-carbon charge densities with the nitronyl-H chemical shifts of nitrones **1**, **2**, **6**-**8**, **10** and **11** ($R^2 = 0.656$) excluding nitrone **9**.

UV-Vis stopped-flow kinetics. We then applied the UV-Vis stopped-flow technique for the determination of the rate constant of $O_2^{\bullet-}$ reaction to nitrones. Phenol red was used as a probe to measure $O_2^{\bullet-}$ production, and the rate of formation of this new species at 575 nm is directly proportional to the kinetics of $O_2^{\bullet-}$ decay.[13, 20] The kinetic values obtained using the stopped-flow technique were shown to correlate well with the trends in rate constants obtained using the EPR technique. The slope of the linear line generated from the UV-Vis absorption plot was used to calculate the rate of nitrone-spin trapping by using equation 1, where N is the PBN derivative, *V* and *v* are the initial rates of $O_2^{\bullet-}$ addition to phenol red (PR) in the absence and presence of PBN derivatives, respectively.

$$V/v - 1 = k_{sN}[N] / k_{PR}[PR] \qquad (1)$$

Table 2.1.5. Relative Rate Constants for $O_2^{\bullet-}$ and $Ph^{\bullet}$ Adduct Formation

Nitrones	**UV-Vis.**[a]		**EPR**[b]	
	k_{sN}/k_{PR} (10^{-3})	k_{sN}/k_{sPBN}	k_{pN}/k_{TN} (± 0.05)	k_{pN}/k_{pPBN}
PBN-CH_2OH (**1**)	16.7 ± 0.6	12.8	0.09	0.68
PBN-CH_2OAc (**2**)	73.6 ± 0.7	56.6	0.18	1.37
PBN-$CH_2OCONHMe$ (**6**)	19.1 ± 0.3	14.7	0.22	1.66
PBN-CH_2NHAc (**7**)	13.8 ± 0.2	10.6	0.27	2.01
PBN-$(CH_2OH)_2$ (**8**)	11.7 ± 0.4	9.0	0.11	0.80
PBN-$(CH_2OH)_3$ (**9**)	14.2 ± 0.1	10.9	nd[c,d]	nd[c,d]
PBN-$(CH_2OAc)_2$ (**10**)	nd[c]	nd[c]	0.37	2.79
PBN-$(CH_2OAc)_3$ (**11**)	nd[c]	nd[c]	0.26	1.94
PBN	1.3 ± 0.0	-	0.13	-
DMPO	17.6 ± 0.5	13.5	nd[c]	nd[c]
TN	nd[c]	nd[c]	-	7.58

[a]Ratio of the second order rate constants for the superoxide radical reaction with various nitrones (k_{sN}) and by PBN (k_{sPBN}) in DMF/KO_2. [b]Ratio of the second order rate constants for the phenyl radical trapping by various nitrones (k_{pN}) and by PBN (k_{pPBN}) in benzene. [c]Not determined. [d]The EPR signal of the adduct **9**-Ph was too weak to allow a reliable determination of the ratio.

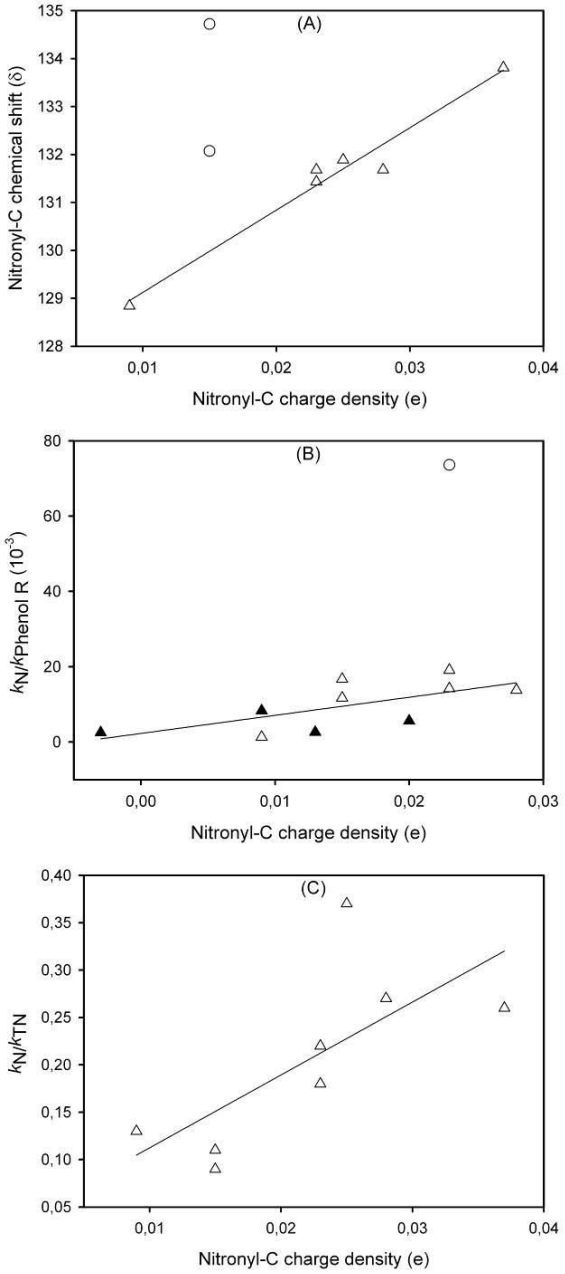


Figure 2.1.10. Correlation of the nitronyl-carbon charge densities with: (A) Nitronyl-C chemical shifts of nitrones **2, 6, 7, 10, 11** and **PBN** (R^2 = 0.965) excluding nitrones **1** and **8** marked as (○) and **9** which is not soluble in $CDCl_3$. (B) Experimental relative rate constant of $O_2^{\bullet-}$ addition to nitrones (k_{sN}/k_{PR}) including para-substituted nitrones marked as (▲) from Durand et al, 2008 (R = 0.451) and excluding nitrone **2** marked as (○). (C) Experimental relative rate constant of phenyl addition to nitrones (k_{pN}/k_{TN}) (R = 0.504).

The slopes of the KO_2 controls were averaged to give k_{PR}. The relative rate constants (k_{sN}/k_{PR}) are shown in Table 3, and for comparison, the rate of formation of DMPO and PBN were

included. All of the relative rates were significantly lower than 1, demonstrating that $O_2^{\bullet-}$ reacts faster with phenol red than with the nitrone spin-traps. From these values, k_{sN}/k_{sPBN} ratio was calculated leading to the following order of increased reactivity to $O_2^{\bullet-}$: PBN-$(CH_2OH)_2$ < PBN-CH_2NHAc < PBN-$(CH_2OH)_3$ < PBN-CH_2OH < DMPO < PBN-$CH_2OCONHMe$ < PBN-CH_2OAc. It is worth mentioning that all substituted nitrones exhibit ~ 10-50 times higher rates of trapping compared to PBN. As shown in Figure 2.1.10.B, the plot of the rate constant of $O_2^{\bullet-}$-nitrone reaction with the nitronyl-C charge density shows increased rates of reaction for the more positively charged carbons, however, with a fairly poor correlation coefficient. For the sake of comparison, we also plotted the data for para-substituted nitrones.[20] The correlation for para-substituted nitrones is even weaker, which made us to conclude in our previous work that $O_2^{\bullet-}$ addition to nitrone might be weakly electrophilic. With more compounds included in this study, the trend may suggest a nucleophilic nature of $O_2^{\bullet-}$ addition to this set of nitrones although the correlation is not satisfactory. This may also suggest that the reaction of $O_2^{\bullet-}$ to nitrone is not charge-controlled but rather orbital-controlled, hence, warrants further investigation.

Spin trapping kinetics. Since some nitrones in the series were poorly water-soluble or were found to be highly reactive towards $HO^{\bullet}$, the use of a Fenton system was precluded. Therefore, we chose to study phenyl radical ($Ph^{\bullet}$) trapping in benzene where the corresponding adducts show high stability. The 1,3,5-tri[(N-(1-diethylphosphono)-1-methylethyl) *N*-oxy-aldimine] benzene (TN)[29] was used as competitive scavenger to examine the relative rates of trapping by the nitrones **1, 2, 6-11** compared to PBN (Figure 2.1.11). It is worth noting that the adduct decay must be slow enough to be neglected to obtain reliable results with this approach.[30] The $Ph^{\bullet}$ was generated by UV photolysis of a solution containing large excess of iodobenzene in the presence of TN and of the nitrone of interest, denoted as N. As previously observed,[29] the possibility of multiple-trapping by TN was

neglected since the poly-adducts were never observed by EPR in our study. In this method, the $Ph^{\bullet}$ spin trapping rate was monitored by measuring the intensity (as the signal area) of the EPR signal of the corresponding adducts. The standard kinetic competition model employed as described elsewhere [29] yielded equation 2. In this equation, the second-order rate constants for $Ph^{\bullet}$ trapping by the nitrone N and TN are denoted as k_{pN} and k_{TN} respectively, while *r* and *R* represent the trapping rate by TN only in presence of N, and by both TN and N, respectively.

$$R/r = 1 + k_{pN}[N] / k_{TN}[TN] \qquad (2)$$

By plotting the *R*/*r* ratio as a function of the [N]/[N]ratio for each nitrone **1, 2, 6-11**, a straight line was obtained with a slope equal to k_{pN}/k_{TN}. Five experiments were performed at five different [N]/[TN] ratios kept between 1 and 4. The commercially available PBN was then employed instead of N in order to determine the k_{pPBN}/k_{TN} ratio. From these results, the k_{pN}/k_{pPBN} ratio was calculated and the values obtained for nitrones **1, 2, 6-11** are reported in Table 2.1.5.

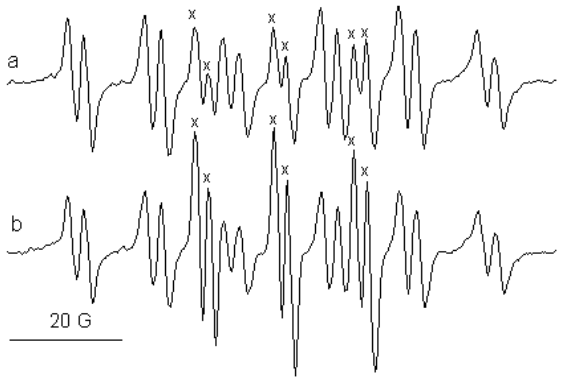


Figure 2.1.11. EPR signals recorded in benzene by photolysis of 3 $mol.L^{-1}$ phenyliodide solution in the presence of the nitrones **10** and **7** at two different ratios [**10**]/[TN]: a) [**10**]/[TN] = 0.67 ([**10**] = 20 $mmol.L^{-1}$ and [TN] = 30 $mmol.L^{-1}$); b) [**10**]/[TN] = 2 ([**10**] = 40 $mmol.L^{-1}$ and [TN] = 20 $mmol.L^{-1}$). The peaks with cross (×) correspond to the phenyl radical adduct of **10**, while the other lines correspond to the phenyl radical adduct of TN.

Though all the nitrones studied were found less efficient than the tri-(phosphorylated nitrone) TN, many of the substituted PBN trapped Ph$^{\bullet}$ significantly faster than PBN where only the hydroxylated compounds exhibited slower trapping rates than PBN. It should be mentioned that the EPR signal of the phenyl radical adduct on **9** was much too weak to permit a reliable evaluation of k_{pN}/k_{TN}. This could be due to a much lower trapping efficiency of **9** and/or to a more rapid decay of the spin-adduct. Actually, it turned out that the Ph$^{\bullet}$ adducts of the hydroxylated nitrones exhibited faster decay than those of other nitrones, and this was more particularly evident in the case of **9**. This observation suggests that the hydroxyl groups would intervene in the nitroxide decay mechanism. The most efficient compounds in the series for trapping Ph$^{\bullet}$ are the di- and tri-acetylated nitrones **10**, and **11** and the amide derivative **7**, while the least effective were PBN and the di- and monohydroxylated nitrones **1** and **7**. Similar to what was observed for the stopped-flow kinetics experiments, the rates of reaction correlate with the nitronyl-C charge density where reasonable correlation can be observed with Ph$^{\bullet}$ (Figure 2.1.10.C). The increased rate of trapping by β-substituted nitrones with increasing positive nitronyl-C charge density suggests a nucleophilic nature of the Ph$^{\bullet}$ addition to the nitronyl-carbon atom. It has been shown that electron-withdrawing substituent on the aromatic ring of PBN-type compounds increases the reactivity of the nitronyl group for nucleophilic addition reactions and nucleophilic radical addition.[31, 32] On the contrary, PBN-type nitrones bearing an electron-donating substituent have also been suggested to exhibit high reactivity to electrophilic radicals.[33, 34] In this work the polar effect of the *N*-*tert*-butyl substituents is obviously electron-withdrawing with hydroxyl, ester, amide and urethane groups, which therefore may favour nucleophilic addition. This is in agreement with the findings by Sueishi *et al*., who suggested the nucleophilic nature of phenyl radical addition to nitrones.[31] More recently De Vleeshouwer et al. confirmed the moderate nucleophilic character of phenyl radical using natural population analysis.[35]

Thermodynamics of adduct formation. Examination of the optimized structures at the B3LYP/6-31G(d) level of theory of various spin-adducts show $C_{nitronyl}$-N and N-O bond distances of 1.280-1.292 Å and 1.472-1.513 Å, respectively, and are consistent with the X-ray crystallographic $C_{nitronyl}$-N and N-O bonds observed for the phenyl radical adduct of *N*-tert-butyl-α-(2-pyridyl)nitrone with bond distances of 1.287-1.291 **Å** and 1.462-1.466 **Å**, respectively.[28] Table 4 shows the energetics of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ addition to various mono-, di-, and tri-, α- and β-substituted PBN derivatives. Majority of the nitrones (22 out of 35) exhibited decreased reactivity of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ as the number of substitution increases from mono-to tri-substitution (Table 4). Only in few cases were the favorability of radical addition significantly increases with increasing substitution (*i.e.,* from mono- to tri-substitution) such as in the addition of $O_2^{\bullet-}$ to –COOH, -NHC(=O)Me, $-CH_2OC(=O)Me$, and $-CH_2SMe$, and $HO_2^{\bullet}$ to –COOH, -NHC(=O)Me, and $-CH_2C(=O)NH_2$. Reactivity of $O_2^{\bullet-}$ in general are endoergic with -NHC(=O)Me mono-, di-and tri-substitution to be the most favorable with $\Delta G_{rxn,\ 298K}$ (kcal/mol) of 12.7, 11.9 and 6.9, respectively. Structure of these $O_2^{\bullet-}$ adducts shows intramolecular H-bonding interaction between the amide-H and peroxyl-O (Figure 2.1.13) resulting in proton abstraction of one of the amide-H's by the peroxide-O to form the hydroperoxyl moiety, similar to that observed for the 5-carbamoyl-5-methyl-1pyrrroline N-oxide (AMPO)[36] with an endoergic $\Delta G_{rxn,\ 298K}$ of 6.1 kcal/mol, and with diamide-substituted DMPO derivatives with $\Delta G_{rxn,\ 298K}$ = -3.3 kcal/mol both of which became the basis for the fast and favorable reactivity of amide-conjugated nitrones compared to other spin-traps with no such strong intramolecular interactions.[13, 14] The reactivity of $HO_2^{\bullet}$ to nitrones that are mono-substituted with $-CH_2OC(=O)Me$ and di-substituted with -OC(=O)Me gave the most exoergic free energies of reaction with $\Delta G_{rxn,298K}$ (kcal/mol) of -4.4 and -3.5, respectively. However, the most exoergic $\Delta G_{rxn,298K}$(kcal/mol) value observed among all the tri-substituted analogues was only -1.0 for the –OMe tri-substitution. Previously, at the same level of theory,

we showed that addition of $HO_2^{\bullet}$ to monoester-substituted, EMPO, and the diester-substituted, DEPO, gave the most exoergic $\Delta G_{rxn,298K}$ of -6.2 kcal/mol compared to other DMPO-derivatives. This suggests that ester conjugation to nitrones are preferred for $HO_2^{\bullet}$ trapping.[37]

Table 2.1.6. Free energies ($\Delta G_{rxn,298K,aq}$ in kcal/mol) of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ addition to *N-tert*-butyl-substituted PBN derivatives at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory in water.

Nitrones		Free energies of radical addition (kcal/mol)					
		$O_2^{\bullet-}$ addition			$HO_2^{\bullet}$ addition		
		mono-	di-	tri-	mono-	di-	tri-
PBN	$-CH_3$	18.3	n/a	n/a	-0.9	n/a	n/a
α-substitution	-COOH	16.8	13.1	7.5	1.7	0.5	0.1
	-C(=O)OMe	16.3	21.9	17.2	1.9	0.5	3.1
	$-C(=O)NH_2$	15.2	14.1	14.8	4.5	4.0	3.4
	-OMe	17.7	19.3	18.3	-3.1	-2.9	-1.0
	-OC(=O)Me	15.8	15.2	[a]	-1.4	-3.5	2.6
	-OC(=O)NHMe	16.7	20.1	21.2	3.6	-2.7	2.0
	-NHC(=O)Me	12.7	11.9	6.9	0.4	0.9	0.6
	-SMe	17.2	22.4	18.3	-2.2	2.0	0.0
	$-P(=O)(OMe)_2$	20.0	28.1	24.5	-0.9	0.4	3.0
β-substitution	$-CH_2OH$	16.1	19.5	20.3	0.8	3.0	2.6
	$-CH_2OC(=O)Me$	16.7	24.7	12.3	-4.4	1.5	1.7
	$-CH_2NHC(=O)Me$	18.0	24.6	16.0	3.4	4.9	1.0
	$-CH_2OC(=O)NHMe$	13.9	17.4	22.6	-2.5	1.1	1.3
	$-CH_2C(=O)NH_2$	17.1	14.8.	15.1	3.0	5.5	-0.7
	$-CH_2C(=O)OMe$	17.8	20.4	18.3	0.9	5.0	-0.3
	$-CH_2OMe$	19.8	17.5	21.7	0.2	1.0	1.1
	$-CH_2P(=O)(OMe)_2$	16.1	23.6	16.8	1.0	0.8	2.4
	$-CH_2SMe$	19.7	18.6	10.3	-0.2	0.9	0.6

[a]After several attempts to optimize this adduct, only fragmented products were obtained.

The absence of a significant global trend in $HO_2^{\bullet}/O_2^{\bullet-}$ reactivity as a function of increasing nitronyl-C charge density and/or increasing substitution could be accounted for the competing inductive effects of the methyl group in the partially α– and β-substituted PBN as well as the competing resonance effect by the phenyl ring with the inductive effect of the α– and β-substitution. For mono-α–substituted PBN (with the exception of two outliers, $-P(=O)(OMe)_2$, -NH(C=O)Me), a fairly poor correlation ($R^2 = 0.473$) can be observed between

the charge densities on the nitronyl-C with their respective free energies ($\Delta G_{rxn,298\ K}$) of addition with $O_2^{\bullet-}$ (Figure 2.1.9).

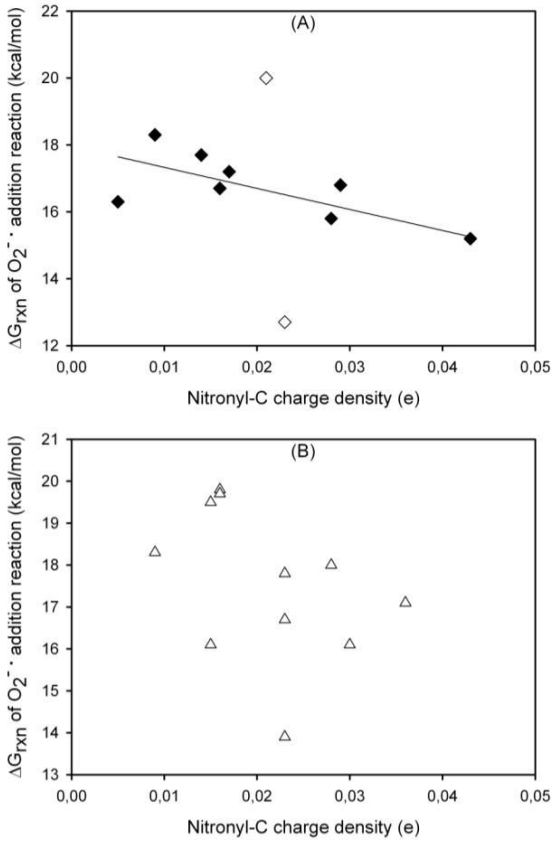
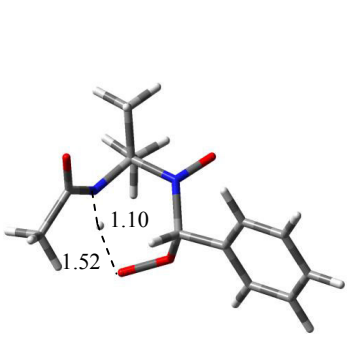


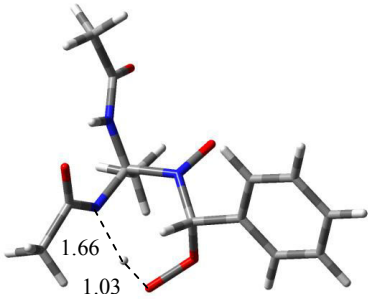
Figure 2.1.12. Plots of NPA charge densities of the nitronyl carbon of the monosubstituted nitrones versus their free energies ($\Delta G_{rxn,\ 298K}$) of $O_2^{\bullet-}$ addition reactions: (A) α-substituted nitrones (◆) (R = 0.473) excluding -P(=O)(OMe)$_2$ and -NHC(=O)Me derivatives (◇). (B) β-substituted nitrones (△) (R = 0.169).

In a similar study involving para-substitution on PBN, we showed that there was no correlation that can be observed for $\Delta G_{rxn,298\ K}$ and nitronyl-carbon charge densities in both $O_2^{\bullet-}$ and $HO_2^{\bullet}$ addition reactions.[20] Therefore, reactivity of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ to mono-α-substituted PBN might be similar in nature (*i.e.*, nucleophilic) to those observed for the $O_2^{\bullet-}$ addition to 5-substituted DMPO analogues which are also mono-α-substituted ones,[13] but the effect is much less pronounced indicating that inductive effect of the substituents is weaker for PBN than that of DMPO analogues. For the mono- β-substitution, no correlation

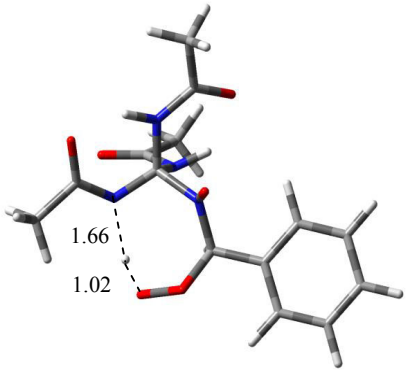
were be observed with the charge density and their respective $\Delta G_{rxn,298K}$ of reactivity to $O_2^{\bullet-}$ (Figure 2.1.12) which could be due to the presence of the methylene group that can further diminish the inductive effect by the functional substituent groups.



PBN-mono-NHC(=O)Me



PBN-di-NHC(=O)Me



PBN-tri-NHC(=O)Me

Figure 2.1.13. Optimized $O_2^{\bullet-}$ adduct structures with the least endoergic free energies of formation showing the intramolecular H-bond interaction of the amide-H with the peroxyl-O.

Cell culture and viability studies. Cytoprotective properties of nitrones against H_2O_2-induced cell death was investigated *in vitro* using bovine aortic endothelial cells (BAEC). BAEC were pre-incubated with varying concentrations (10-50 μM) of nitrone derivatives **1**, **2**, and **6-8** (*i.e.*, PBN-CH_2OH, PBN-CH_2OAc, PBN-$CH_2OCONHMe$, PBN-CH_2NHAc, and PBN-$(CH_2OH)_2$) and were challenged for two hours with H_2O_2 (1 mM). Extent of cytoprotection was measured using [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium-bromide] (MTT) assay. Our results are presented in Figure 2.1.14 and show that at 10 μM nitrones **2** and **7** exhibited the highest cytoprotection against H_2O_2-induced toxicity. At 50 μM, the protection afforded by the nitrones was more pronounced for PBN and compounds **1**, **7** and **8** while for compounds **2** the protection remained similar. A significant decrease of cell viability for compound **6** was noted which was even lower than the control and may indicate a slight toxicity due to the urethane substituent.

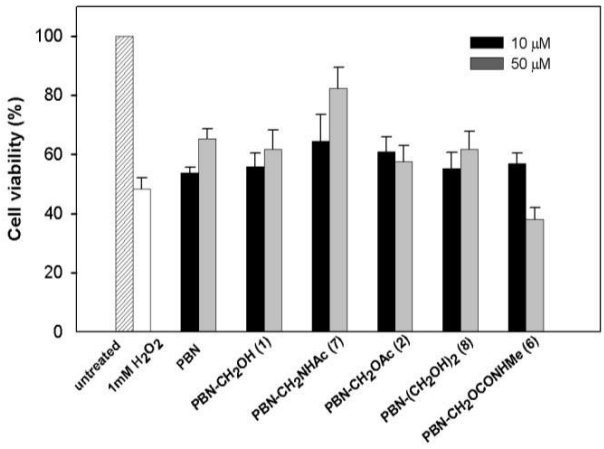


Figure 2.1.14. Cytoprotectivity of PBN derivatives at 10 and 50 μM on Bovine Aortic Endothelial Cells against 1 mM H_2O_2 after 2 hours of incubation.

To examine the relationship between the electrochemical properties and the antioxidant activity of our derivatives, we tried to correlate the cytoprotection data versus the electrochemical potentials of the nitrones. While no correlation between the reduction potential and the cell viability of nitrone-treated BAEC was observed, a good correlation was

observed for the oxidation potentials recorded in acetonitrile with the following order of increasing protective property: PBN-$CH_2OCONHMe$ < PBN-CH_2OAc < PBN-CH_2OH ~ PBN-$(CH_2OH)_2$ < PBN < PBN-CH_2NHAc. Whereas this correlation is particularly obvious at 50 μM where increased cytoprotection is inversely correlated with the oxidation potential (Figure 2.1.15), at 10 μM no correlation was observed (data not shown). It has to be noted that at 10 μM, the protection afforded by the nitrones against 1 mM H_2O_2 was very limited. This indicates that the ability of nitrones to be oxidized at lower potentials offers better cytoprotection, further supporting the role of nitrones in attenuating oxidant-mediated toxicity by an antioxidant mechanism and not solely through spin trapping properties.

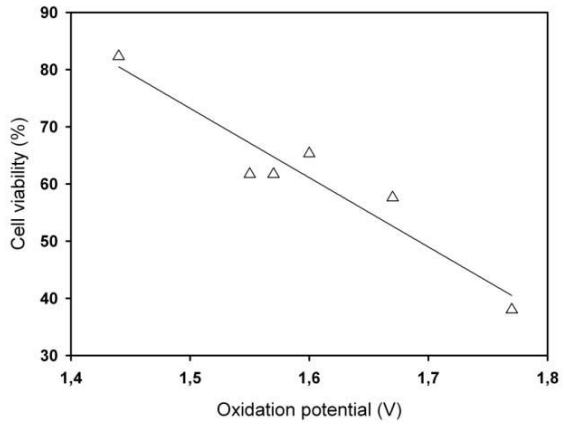


Figure 2.1.15. Correlation of cell viability with oxidation potential of nitrones **1**, **2**, **6-8** and **PBN** in acetonitrile ($R^2 = 0.910$).

III. Conclusion

In this work, we have studied the electronic effect of various substituents on the reactivity of α-phenyl *N-tert*-butyl nitrones. A series of *N-tert*-butyl substituted mono-hydroxyl (CH_2OH); mono-ester (CH_2OAc); mono-amide (CH_2NHAc) and mono-urethane ($CH_2OCONHMe$) as well as di- and tri-hydroxyl and ester derivatives was prepared in good yield. The substituent effect on the redox properties was investigated by cyclic voltammetry and showed that electron-withdrawing groups make the nitronyl group more difficult to oxidize. The

substituent effect was also demonstrated by computational approach where increased positivity on the nitronyl-carbons were observed for multiple α- and β-substituted compounds, which correlates well with experimental NMR chemical shifts. A UV-Vis stopped-flow kinetic technique was used to demonstrate the nucleophilic nature of superoxide ($O_2^{\bullet-}$) addition to nitrone in agreement with previous findings on cyclic nitrones. The thermodynamics of $O_2^{\bullet-}$ adduct formation showed that the reactivity is endoergic in general, however for α-substituted derivatives, a modest correlation was observed with the nitronyl charge density suggesting a weak nucleophilic nature of $O_2^{\bullet-}$ addition. Moreover, the nucleophilic nature of phenyl radical ($Ph^{\bullet}$) addition to nitrone was also observed using EPR kinetic method. Finally, a correlation between the cytoprotective property of nitrones against H_2O_2-induced cell death and their oxidation potential was observed indicating the antioxidant properties is also affected by the nature of the substituent. This study confirms that the electronic effect of the substituents grafted on the *N*-*tert*-butyl group is of high importance in the design of nitrone with improved trapping and antioxidant properties. Among the nitrones tested, the amide derivative PBN-CH_2NHAc gave the best properties such as low oxidation potential, good trapping properties combined with cytoprotective activity making the amide bond an efficient linker for *N*-*tert*-butyl functionalization of the α-phenyl *N*-*tert*-butyl nitrone.

IV. Experimental Section

Synthesis. All reagents were from commercial sources and were used as received. All solvents were distilled and dried according to standard procedures. TLC analysis was performed on aluminum sheets coated with silica gel (40-63 μm). Compound detection was achieved either by exposure to UV light (254 nm) and by spraying a 5% sulphuric acid solution in ethanol or a 2% ninhydrin solution in ethanol, and then by heating at ~ 150°C. Flash chromatography was carried out on silica gel (40-63μm). Size exclusion

chromatography was carried out on hydroxypropylated cross-linked dextran. UV/vis spectra were recorded on UV/vis spectrometer aquipped with a double-compartment quartz cell of 10-mm length.. Melting points have not been corrected. The ^{1}H NMR spectra were recorded at 250 or 400 MHz and the ^{13}C NMR at 62.86 or 100 MHz. Chemical shifts are given in ppm relative to the solvent residual peak as a heteronuclear reference for ^{1}H and ^{13}C. Abbreviations used for signal patterns are: bs, broad singulet; s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet. HR-MS spectra were recorded on a mass spectrometer equipped with a TOF analyzer for ESI + experiments.

α-Phenyl-N-(2-methyl-1-hydoxy-2-propyl)nitrone (1). In an argon atmosphere and under stirring, benzaldehyde (0.80 g, 7.54 mmol), 2-methyl-2-nitro-1-propanol (1.8 g, 15.1 10^{-3} mol) and AcOH (2.5 mL, 45.08 mmol) were dissolved in EtOH. The mixture was cooled down to 0°C then zinc powder (1.92 g, 29.6 mmol) was slowly added in order to keep the temperature below 15°C. The mixture was stirred at room temperature for a couple of minutes then heated at 60°C in the dark for 10 h in the presence of molecular sieves (4 Å). The reaction mixture was filtered off through a pad of Celite, and the solvent was removed under vacuum. The crude mixture was purified by flash chromatography (EtOAc/cyclohexane 6:4 v/v) followed by two successive crystallizations from EtOAc/*n*-hexane to give compound **1** (0.94 g, 4.87 mmol, 65%) as a white powder. *Rf* 0.42 (EtOAc/cyclohexane 8:2 v/v); mp 78.5-78.9°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.27 (2H, m), 7.48 (1H, s), 7.43 (3H, m), 4.24-4.27 (1H, t, J = 6.2 Hz), 3.79-3.80 (2H, d, J = 6.0 Hz), 1.61 (6H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 132.1, 130.7 (CH), 130.3 (C), 129.2 (CH), 128.5, 72.9 (C), 70.0 (CH_2), 23.9 (CH_3); UV (MeOH) λ_{max} 296 nm; HR-MS (ESI+, m/z) calcd for $C_{11}H_{15}NO_2$ [$(M+H)^+$]: 194.1181, found 194.1180. The spectra data of compound **1** were in agreement with those reported by Janzen et al. except for the melting point that was found to be 75-76°C.[16]

α-Phenyl-N-(2-methyl-1-acetate-2-propyl)nitrone (2). Under stirring, compound **1** (0.35 g, 1.81 mmol) was dissolved in a Ac_2O/pyridine (1:1 v/v) mixture at 0°C. After 12h of stirring at room temperature, the mixture was poured into cold 1 N HCl and extracted with CH_2Cl_2 (3×). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The crude mixture was purified by flash chromatography (cyclohexane/EtOAc 6:4 v/v) to give compound **2** (0.42 g, 1.79 mmol, 98%) as a white oil. *Rf* 0.38 (EtOAc/cyclohexane 5:5 v/v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.30 (2H, m), 7.50 (1H, s), 7.43 (3H, m), 4.43 (2H, s), 2.03 (3H, s), 1.62 (6H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5 (CO), 130.7 (C), 130.5, 129.0, 128.5 (CH), 72.1 (C), 68.3 (CH_2), 23.7, 20.8 (CH_3); UV (MeOH) λ_{max} 298 nm; HR-MS (ESI+, m/z) calcd for $C_{13}H_{17}NO_3$ [$(M+H)^+$]: 236.1286, found 236.1283.

2-methyl-2-nitropropyl methylcarbamate (3). In an argon atmosphere and under stirring in a sealed tube, 2-methyl-2-nitro-1-propanol (1 g, 8.39 mmol), DCI (2.11 g, 16.78 mmol) and DMAP (0.102 g, 0.839 mmol) were dissolved in THF under argon atmosphere. After 2 h of stirring at room temperature, methylamine (1.13 g, 16.78 mmol) was added and the stirring was continued for 18 h. Then, the mixture was filtered and the solvent was removed under vacuum. The crude mixture was purified by flash chromatography (cyclohexane/EtOAc 9:1 v/v) to give compound **3** (1.45 g, 8.23 mmol, 98%) as a white powder. *Rf* 0.30 (cyclohexane/EtOAc 8:2 v/v); mp 48.3-49.3°C; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 4.76 (1H, bs), 4.39 (2H, s), 2.78 (3H, d, *J* = 4.90 Hz), 1.59 (6H, s); ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 156.0 (CO), 86.7 (C), 68.7 (CH_2), 27.6, 23.0 (CH_3). HR-MS (ESI+, m/z) calcd for $C_6H_{13}N_2O_4$ [$(M+H)^+$]: 177.0875, found 177.0878.

2-methyl-2-nitro propanamide (5). The synthetic procedure was essentially the same as for compound **2**. 2-methyl-2-nitro propanamine[38](3.70 g, 31.50 mmol) was used as starting material. The crude mixture was purified by flash chromatography (EtOAc/cyclohexane 8:2 v/v) to give compound **5** (4.7 g, 29.16 mmol, 94%) as a white powder. *Rf* 0.48 (EtOAc); mp

102.6-103.1°C; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 6.15 (1H, bs), 3.71 (2H, d, *J* = 6.6 Hz), 2.00 (3H, s), 1.56 (6H, s); ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 170.7 (CO), 88.8 (C), 46.1 (CH_2), 24.0, 23.2 (CH_3). HR-MS (ESI+, m/z) calcd for $C_6H_{13}N_2O_3$ [$(M+H)^+$]: 161.0926, found 161.0927.

α-Phenyl-N-(2-methyl-1-methylcarbamate-2-propyl)nitrone (6). The synthetic procedure was essentially the same as for compound **1**. Benzaldehyde (0.30 g, 2.84 mmol) and compound **3** (1 g, 5.67 mmol) were used as starting materials. The crude mixture was purified by flash chromatography (EtOAc/cyclohexane 5:5 v/v) followed by two successive crystallizations from EtOAc/*n*-hexane to give compound **6** (0.5 g, 2.0 mmol, 70%) as a white powder: *Rf* 0.23 (EtOAc/cyclohexane 7:3 v/v); mp 127.8-128.1°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.30 (2H, m), 7.48 (1H, s), 7.42-7.43 (3H, m), 4.66 (1H, m), 4.45 (2H, s), 2.76-2.77 (3H, d, *J* = 4.9 Hz), 1.60 (6H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.6 (CO), 131.4 (CH), 130.7 (C), 130.4, 128.9, 128.5 (CH), 72.6 (C), 68.5 (CH_2), 27.6, 23.6 (CH_3); UV (MeOH) λ_{max} 298 nm; HR-MS (ESI+, m/z) calcd for $C_{13}H_{18}N_2O_3$ [$(M+H)^+$]: 251.1395, found 251.1390.

α-Phenyl-N-(2-methyl-1-acetamide-2-propyl)nitrone (7). The synthetic procedure was essentially the same as for compound **1**. Benzaldehyde (0.26 g, 2.45 mmol) and compound **5** (0.8 g, 5.0 mmol) were used as starting materials. The crude mixture was purified by flash chromatography (EtOAc) followed by two successive crystallizations from EtOAc/*n*-hexane to give compound **7** (0.39 g, 1.66 mmol, 68%) as a white powder. *Rf* 0.35 (EtOAc/methanol 9.5:0.5 v/v); mp 114.1-114.6°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.27 (2H, m), 7.50 (1H, s), 7.44 (3H, m), 6.62 (1H, m), 3.68-3.70 (2H, d, *J* = 6.3 Hz), 1.98 (3H, s), 1.60 (6H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5 (CO), 131.7, 130.7 (CH), 130.4 (C), 129.0, 128.6 (CH), 73.4 (C), 47.3 (CH_2), 25.1, 23.3 (CH_3); UV (MeOH) λ_{max} 298 nm; HR-MS (ESI+, m/z) calcd for $C_{13}H_{18}N_2O_2$ [$(M+H)^+$]: 235.1446, found 235.1442.

α-Phenyl-N-(2-methyl-1,3-dihydroxy-2-propyl)nitrone (8). The synthetic procedure was essentially the same as for compound **1**. Benzaldehyde (0.78 g, 7.40 mmol) and 2-methyl-2-nitro-1,3-propanediol (2 g, 14.80 mmol) were used as starting materials. The crude mixture was purified by flash chromatography (EtOAc) followed by two successive crystallizations from EtOAc/*n*-hexane to give compound **8** (0.99 g, 4.74 mmol, 65%) as a white powder. *Rf* 0.21 (EtOAc); mp 84.2-85.6°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.24 (2H, m), 7.52 (1H, s), 7.43 (3H, m), 3.91-3.98 (6H, m), 1.50 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 134.7, 131.1 (CH), 129.9 (C), 129.6, 128.6 (CH), 75.9 (C), 66.8 (CH_2), 19.3 (CH3); UV (MeOH) λ_{max} 296 nm; HR-MS (ESI+, m/z) calcd for $C_{11}H_{15}NO_3$ [$(M+H)^+$]: 210.1130, found 210.1126. The spectra data of compound **8** were in agreement with those reported by Janzen et al.[16] except for the melting point that was found to be 52-55°C.

α-Phenyl-N-(2-methyl-1,3-di-O-acetyl-2-propyl)nitrone (10). The synthetic procedure was essentially the same as for compound **2**. Compound **8** (0.40 g, 1.79 mmol) was used as starting material. The crude mixture was purified by flash chromatography (cyclohexane/EtOAc 4:6 v/v) to give compound **10** (0.25 g, 0.85 mmol, 45%) as a white oil. *Rf* 0.27 (EtOAc/cyclohexane 6:4 v/v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.22 (2H, m), 7.41 (1H, s), 7.36 (3H, m), 4.50 (2H, d, *J* = 11.7 Hz), 4.35 (2H, d, J = 11.7 Hz), 1.98 (6H, s), 1.59 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.2 (CO), 131.9, 129.8 (CH), 129.3 (C), 128.1, 127.5 (CH), 72.9 (C), 64.3 (CH_2), 19.7, 17.8 (CH_3); UV (MeOH) λ_{max} 298 nm; HR-MS (ESI+, m/z) calcd for $C_{15}H_{19}NO_5$ [$(M+H)^+$]: 294.1341, found 294.1337.

α-Phenyl-N-(2-O-acetylmethyl-1,3-di-O-acetyl-2-propyl)nitrone (11). The synthetic procedure was essentially the same as for compound **2**. α-Phenyl-N-(2-hydroxymethyl-1,3-dihydroxy-2-propyl)-nitrone[17] (0.40 g, 1.77 mmol) was used as starting material. The crude mixture was purified by flash chromatography (cyclohexane/EtOAc 6:4 v/v) to give compound **11** (0.43 g, 1.22 mmol, 69%) as a white oil. *Rf* 0.42 (EtOAc/cyclohexane 5:5 v/v);

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.28 (2H, m), 7.45 (3H, s), 7.34 (1H, m), 4.61 (6H, s), 2.06 (9H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.9 (CO), 133.9, 131.1 (CH), 130.0 (C), 129.3, 128.6 (CH), 75.5 (C), 61.4 (CH_2), 20.6 (CH_3); UV (MeOH) λ_{max} 300 nm; HR-MS (ESI+, m/z) calcd for $C_{17}H_{21}NO_7$ [$(M+H)^+$]: 352.1396, found 352.1388.

Determination of water solubility. For PBN and nitrones **6** and **9**, a UV-calibration curve at 290 nm was established from solutions ranging from 10^{-3} to 10^{-2} g/L ($R > 0.995$). A saturated solution of nitrone was prepared at 40°C and then let stand at RT overnight. After centrifugation (12000 g – 15 minutes) at room temperature, the concentration of the supernatant solution was determined using the calibration curve. For nitrones **1**, **7** and **8**, weighted amounts of the nitrone were added into a vial containing water at RT. After each addition, the solution was carefully shaken and the complete dissolution was checked by visual observation.

Determination of log k'_W values. Compounds were dissolved in MeOH at 1.0 mg/mL and were injected onto a C18 reverse phase column (250 mm x 4.6 mm, 5µm). The compounds were eluted at various MeOH and water ratios (7:3 to 3:7 v/v) using a flow rate of 0.8 mL/min. The column temperature was 25°C, and the UV detector wavelength was λ = 298 nm. Linear regression analysis were performed on three data points for compound **9** (from 5:5 to 3:7; $r^2 = 0.9996$); four points for compound **1** (from 6:4 to 3:7; $r^2 = 0.9945$), compound **8** (from 6:4 to 3:7; $r^2 = 0.9973$), compound **2** (from 7:3 to 4:6; $r^2 = 0.9957$), compound **10** (from 7:3 to 4:6; $r^2 = 0.9951$), compound **11** (from 7:3 to 4:6; $r^2 = 0.9976$); five points for compound **7** (from 7:3 to 3:7; $r^2 = 0.9936$) and compound **6** (from 7:3 to 3:7; $r^2 = 0.9944$). The log k' values were calculated by using the equation: $\log k' = \log((t-t_0)/t_0)$, where t is the retention time of the nitrone and t_0 is the elution time of MeOH, which is not retained on the column.

Determination of Clog*P* values. The partition coefficient octanol/water (Clog*P*) was determined using MarvinSketch 5.9.0 that is available at www.chemaxon.com/marvin.

Cyclic Voltammetric Measurement. The electrochemical experiments were carried out using a three-electrode cell in a dry argon atmosphere at room temperature. An Ag/AgCl/saturated NaCl electrode was used as the reference electrode and a platinum wire as the auxiliary electrode. The working electrode (glassy carbon) was polished prior to each experiment using a 0.04 μm aqueous alumina slurry on a wetted polishing cloth.

EPR Measurements. EPR measurements were carried out on a bench EPR spectrometer. The general instrument settings used for spectral acquisition were as follows: microwave power, 10 mW; modulation amplitude, 2 G; received gains, $9 \times 10^1 - 9 \times 10^2$; scan time, 60 s and sweep width, 99, 147 or 249 G. Spectra were recorded at room temperature and measurements were performed using a 50μL quartz cell or capillary tube for UV or non-UV irradiations experiments, respectively. The spectrum simulation was carried out using the WINSIM program [39] available as free software from Public Electron Paramagnetic Resonance Software Tools (http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/).

Spin Trapping Studies. Hydroxyl radical adduct. To generate the hydroxyl radical, nitrone (20 mM) was dissolved in a Fenton system containing hydrogen peroxide (0.2%), EDTA (2 mM), and iron-(II) sulfate (1 mM) in Phosphate Buffer Saline solution. **Superoxide radical adduct.** *KO_2 generating System*. The superoxide anion radical was generated using different concentrations of nitrones (40mM for compound **2** and **6**; 80 mM of compound **10** and 20 mM in other case) to a solution of DMSO containing 20 % of saturated solution of KO_2 in DMSO. *Pyridine/H_2O_2 System*. A pyridine solution of nitrone (20 mM) containing 230 mM H_2O_2 was used. **Methoxy radical adduct.** The methoxy radical was generated by adding ~ 1 mg of solid $Pb(OAc)_4$ to a DMSO solution of nitrone (25 mM) containing 10% v/v of MeOH. **Phenyl radical adduct**. The phenyl radical was generated by photolysis of a benzene

solution of phenyliodide (3mM), using a xenon discharge lamp (250W) giving near UV and visible radiations in the presence of nitrone (50 mM).

General computational methods. For the addition of each radical species ($O_2^{\bullet-}$ or $HO_2^{\bullet}$) to substituted PBN derivatives, density functional theory[40, 41] computational approach was employed to determine the optimized geometry, vibrational frequencies, and single-point energy of all stationary points.[35, 42-44] The effect of aqueous solvation was also investigated using the polarizable continuum model (PCM).[45-49] All calculations were performed using Gaussian 03[50] at the Ohio Supercomputer Center. Single-point energies were obtained at the B3LYP/6-31+G** level[51] based on the optimized B3LYP/6-31G* geometries. Charge and spin densities were obtained from a natural population (NPA)[52] analysis and percent electron localization obtained from natural bond orbital (NBO)[53] analysis at the single point PCM/B3LYP/6-31+G**//B3LYP/6-31G* level. These calculations used six Cartesian d functions. Stationary points for nitrones and its respective adducts have zero imaginary vibrational frequency as derived from a vibrational frequency analysis (B3LYP/6-31G*). A scaling factor of 0.9806 was used for the zero-point vibrational energy (ZPE) corrections for the B3LYP/6-31G* level.[54] Here, thermal correction to Gibbs free energy was added to the total energy, that is, the sum of total electronic (ε_0) and thermal free (G_{corr}) energies with ZPE correction (as outputs from Gaussian) were used for ΔG values estimation at 6-31G* with the solvent effect added at 6-31+G**. The ΔG of reactions were simply the difference of the sums of these values for the reactants and the products. Spin contamination for all of the stationary point of the radical structures was negligible, *i.e.*, $\langle S^2 \rangle$ = 0.75.

Stopped-flow kinetics. Procedure followed similar to Villamena et al.[13] A solution of KO_2 saturated DMF was prepared by adding ~ 200 mg of KO_2 to 5 mL DMF under nitrogen atmosphere. The solution was sonicated and let stand for 5 min. The supernatant (1 mL) was

further diluted with 10 mL DMF to reach a maximum absorbance of ~ 3 at 575 nm when mixed with 500 µM phenol red in 90% DMF – 10% H_2O. This solution was kept on ice and under a nitrogen atmosphere and let stand for 10 min before stopped-flow testing. Solutions of the nitrones and 500 µM phenol red in 90% DMF – 10% H_2O were prepared. A stopped-flow technique consisted of 150 µL KO_2 solution and 150 µL nitrone solution, and the growth and decay of absorption was measured using a UV-vis spectrophotometerrapid mix accessory. The plot was exported to Sigma Plot 11.0 and the absorption increase was fitted to a linear equation (y = ax + b). To ensure a constant concentration of KO_2 throughout the experiment, a control of KO_2 and 500 µM phenol red was performed both before and after nitrone testing. Each nitrone was tested with four or more concentrations ranging from 5 – 200mM.

Spin trapping kinetics. The solvents were of the highest grade of purity commercially available and used without further purification. The trinitrone TN was synthesized and purified as previously described.[29] Phenyl radical was produced directly in the EPR spectrometer cavity by UV photolysis of a 3 $mol.L^{-1}$ iodobenzene solution in benzene. The method of kinetic competition permitted to evaluate the ratio of the second-order rate constants for the trapping of $Ph^{\bullet}$ by one of the nitrone N of interest (k_{pN}, corresponding to the compounds **1**, **2** and **6**-**11**) and TN (k_{TN}), used as competitive inhibitor. Then, the commercially available PBN was also tested versus TN in order to determine the ratio of the rate constants for the trapping of Ph· by PBN and by TN, *i.e.* k_{pPBN}/k_{TN}. The concentration of the various nitrones was varied from 5 to 20 $mmol.L^{-1}$ keeping the [N]/[TN] ratio between 1 and 4. For each nitrone, five experiments were repeated twice at [N]/[TN] values equal to 1, 1.6, 2, 3.2 and 4. In each case, a series of 30 EPR spectra was then recorded (scan time for a single spectrum; 15 s) on a spectrometer operating at X-band with 100 kHz modulation frequency. The signal-to-noise ratio was improved using a SVD procedure, as described elsewhere.[55] The signal recorded exactly 1.5 min after the beginning of the reaction was

then simulated using the winsim software in order to determine the relative areas of the adducts N-Ph and TN-Ph. In this approach, the ratio R/r was evaluated as follows:

$$\frac{R}{r} = \frac{\text{area of N-Ph signal + area of TN-Ph signal}}{\text{area of TN-Ph signal}}$$

Cell culture and viability studies. Bovine aortic endothelial cells (BAEC) were cultured in T-75 flasks, in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L glucose, 10 % Fetal bovine serum, L- glutamine, 2.5 mg/L endothelial cell growth supplement, 1% non-essential amino acids, and 1% pen/strep at 37°C in a humidified atmosphere of 5% CO_2 and 20% O_2. Cells were subcultured after 85-90 % confluence. Cytoprotection of β-substituted nitrones against H_2O_2-induced toxicity was assessed *via* intracellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to its insoluble formazan form. A confluent BAEC culture was seeded onto 96-well plates (~ 1.0 x 10^4 cells/well) and incubated for 24 hrs. BEAC were pretreated with various nitrone concentrations (25 μM, 50 μM, and 100 μM) and incubated for 24 hrs. The cells were then incubated in 1 mM hydrogen peroxide for 2 hrs, followed by the addition of 100 μL phosphate buffered saline (PBS) and 50 μL of MTT solution (5 mg/mL, 5% ethanol) for 1 hr. The cells were then incubated in 200 μL of dimethyl sulfoxide (DMSO) for 2 hrs. Formazan formation was measured using a microplate reader at 595 nm absorbance. Data were calculated as percent absorbance of untreated cells ± SEM (n=5).

Chapter II

Towards the improvement of the intrinsic properties of α-Phenyl-*N*-*tert*-butyl Nitrone

Chapter II – Part 2

Electrochemical and Spin-Trapping Properties of *para*-substituted α-Phenyl-*N*-*tert*-butyl Nitrones

This work will be submitted for publication to *New Journal of Chemistry*: Marie Rosselin, Béatrice Tuccio, Frederick A. Villamena and Grégory Durand.

I. Introduction

Oxidative stress has been implicated in several pathophysiological disorders such as cardiovascular diseases,[56] cancer,[57] stroke and neurodegenerative diseases.[58] The ability of nitrones to prevent oxidative stress-mediated damage in *in-vitro*, *in-vivo* and *ex-vivo* models has made them promising synthetic antioxidants with considerable potential as therapeutics.[5, 59] Within the past twenty years, several nitrone derivatives have been developed among which, one can cite MDL cyclic nitrones[60, 61], azulenyl-based nitrones[62, 63], imidazolyl nitrones [64, 65], α-aryl-*N*-alkyl nitrones[66, 67] as well as conjugate of tetramethylpyrazine[68] to name only a few.

Aside from their use as therapeutic antioxidants, nitrones have been used for spin-trapping experiments where the nitronyl group reacts with a free-radical to form a stable and identifiable aminoxyl radical that is detected by EPR spectroscopy.[69] The spin trapping technique was developed in the late 1960s and since then nitrones have been widely used as analytical reagents to identify biological relevant free radicals such as oxygen-, carbon- or sulfur-centered radicals. Although there are several evidence that support that radical trapping and antioxidant properties of nitrones are not dependent each other, the precise mechanism by which these compounds act in different biological models has not yet been fully elucidated.[4] The nitronyl moiety exhibits electrochemical activity both in aqueous and non-aqueous media. Because of the redox behavior of nitrone spin-traps, "inverted spin-trapping" has been reported.[24] In this process, the nitronyl group is oxidized to its radical cation, or reduced to its radical anion which can then react, respectively, with a nucleophile or an electrophile yielding an aminoxyl radical in both cases. It is important to know the redox behavior of nitrones in order to determine in which conditions spin-trapping reactions occur. In addition, the ease of oxidation of nitrone derivatives has been found to correlate with antioxidant properties.[62, 70] Similar to this, correlation of the ease of reduction of indolone-*N*-oxide

and their anti-malarial activity has been established.[71]

One of strategies in the design of efficient nitrone-based spin-traps is to target the nitronyl group in relevant sites of radical production by conjugating it to specific target ligands. The choice of linker groups for optimal spin-trapping properties is highly desirable [14, 20] and it has been demonstrated that the nature of the linker group also affects its bioactivity.[70] Over the past years, we have explored the reactivity of various *para*- and *N*-*tert*-butyl-substituted phenyl nitrones [20, 70] so as to identify the most optimal linker groups and design selective targeted nitrone-based spin-traps with improved reactivity towards free radicals.

In this work, we report the redox properties of *para*-substituted nitrones (Figure 2.2.1) using cyclic voltammetry in aqueous and organic media. The relationship between the electrochemical behavior of the nitronyl group and the nature of the substituent *i*.*e*. electron-donating *vs*. electron-withdrawing was studied. The rate constant of phenyl radical trapping by these nitrones was next determined using an EPR competition kinetic method.

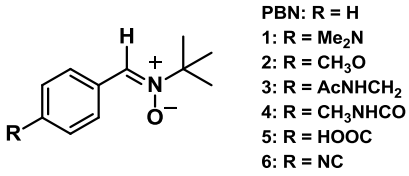


Figure 2.2.1. Chemical structures of the *para*-substituted nitrones used in this work.

II. Results and Discussion

The electrochemical characterization of these nitrones was investigated using cyclic voltammetry and values are reported in Table 2.2.1. We first studied the electrochemical properties of nitrones in acetonitrile containing tetra-butylammonium perchlorate (TBAP) as electrolyte. Aldonitrones are known to undergo a one-electron oxidation in non-aqueous media.[21] For all the nitrones tested, one oxidation potential was clearly observed ranging from +1.29 V to +1.94 V. Only, for Me_2N-PBN (**1**) three distinct peaks were observed (Figure 2.2.2).

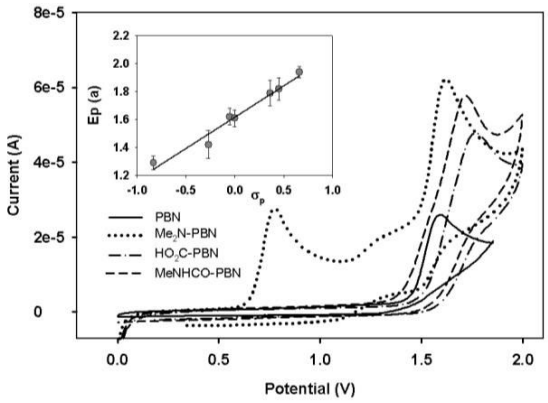


Figure 2.2.2. Oxidation of PBN and compounds **1**, **5** and **6** in acetonitrile containing 50 mM of TBAP at 0.1 $V.s^{-1}$. Inset is the correlation of Hammett sigma para constants (σ_p) with oxidation potentials (R^2=0.97).

C-Anilinonitrones were found to undergo a series of oxidation,[25] which suggests that the two supplementary oxidation potentials at +0.84 V and +1.67 V originates likely from the oxidation of the dimethylaminogroup. A good correlation between the anodic potential and the Hammett sigma para constants (σ_p) of the *para*-substituents was observed (Inset Figure 2.2.2), with compounds bearing electron-withdrawing substituents being hardly oxidized while those bearing electron-donating substituents were more easily oxidized. Looking at the resonance structures shown in Figure 2.2.3, the polar effect from the substituent can therefore be explained by the stabilization of the nitroxide formed after oxidation of the nitronyl group when there is an electron-donating group (A).

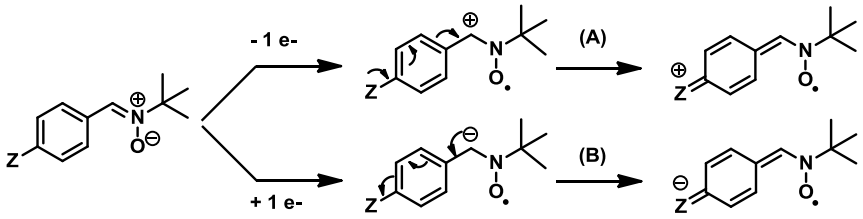


Figure 2.2.3. Oxidation and reduction of PBN and resonance structures in the presence of: (A) an electron-donating group (EDG) and (B) an electron-withdrawing group (EWG).

Reduction of nitrones in acetonitrile was also investigated (Figure 2.2.4). Aldonitrones are known to undergo a one-step two-electron reduction in non-aqueous media.[21] In our hands, for PBN and the nitrones bearing an electron-donating group *i.e*. Me_2N-PBN (**1**), MeO-PBN

(**2**) and $AcNHCH_2$-PBN (**3**), two reductions potentials were observed and whose difference from each other was similar for the four compounds (0.19 ± 0.05 V). The presence of two peaks may indicate the formation of unstable intermediate one-electron reduction species as already suggested by McIntire *et al*. for pyridyl nitrones.[21] On the contrary, for the three nitrones bearing an electron-withdrawing substituent only one reduction peak was observed. This may indicate that an EWG, by stabilizing the intermediate radical anion as shown in Figure 2.2.3.B, results in an easiest overall reduction. For MeNHCO-PBN (**4**) and NC-PBN (**6**), a second peak was also observed however with a difference from that of the nitronyl group of ~ 0.7 V. It is worth noting that in dry acetonitrile although it was not the most common scenario, in some cases only one peak could be observed whose potential was the lower of the two peaks. We therefore considered the lowest reduction potential for the correlation with the Hammett values and a good correlation was noted (Inset Figure 2.2.4), with an increased ease of reduction as the substituent becomes electron-withdrawing, in agreement with the stabilizing effect of the nitroxides as shown in Figure 2.2.3.B. This is in agreement with the usual trend that electron-donating substituents retard the addition of electrons leading to more negative potentials.[25, 72]

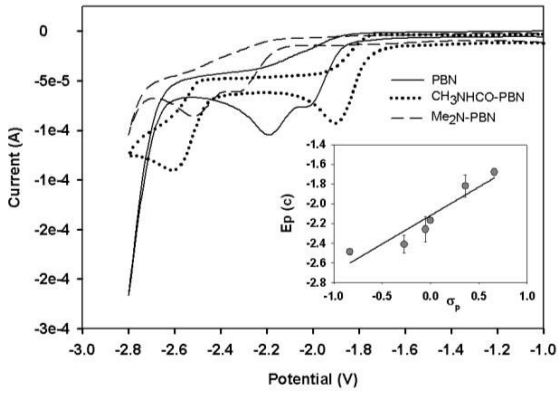


Figure 2.2.4. Reduction of PBN and nitrones **1** and **4** in acetonitrile containing 50 mM of TBAP at 0.1 $V.s^{-1}$ Inset is the correlation of Hammett sigma para constants (σ_p) values with reduction potentials (R^2=0.92).

Voltammograms were recorded with increasing amount of water (Figure 2.2.5). Addition of water from 1% to 30% in volume led to less negative potentials in agreement with the findings by Nepveu and colleagues on *N*-oxide derivatives[71] who suggested that water acting as a proton source could protonate the nitroxide radical anion. For the nitrones exhibiting two reduction peaks, the shift was similar for the two peaks until a certain amount of water (>10%) where they become broader and progressively coalesce. For MeNHCO-PBN (**4**) and NC-PBN (**6**) the single reduction peak of the nitronyl group followed the same trend shifting towards less negative potential values as the water contain increased, while the second peak that was not assigned to the nitrone group was not affected.

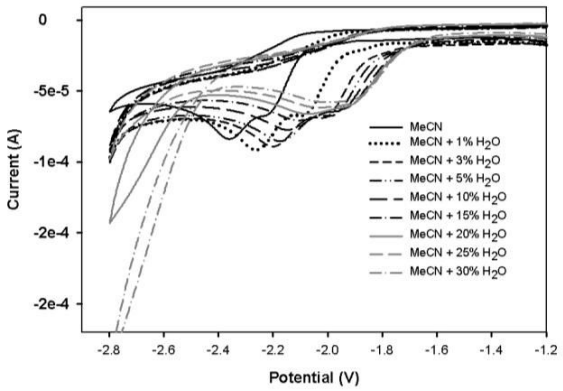


Figure 2.2.5. Reduction of PBN in acetonitrile containing 50 mM of TBAP at 0.1V.s^{-1} with increasing amount of water.

We next carried out cyclic voltammetry in 50 mM NaCl aqueous solution and as already observed no oxidation of the nitronyl group was observed.[21] On the contrary, we observed that all nitrones exhibited an irreversible one-step reduction (Figure 2.2.6). The cathodic peak potential of the PBN derivatives was comprised between -1.41 V and -1.92 V *vs*. Ag/AgCl, that of the PBN being -1.74 V. Along the series as already observed in acetonitrile, compounds bearing an electron-donating group were more hardly reduced than those having an electron-withdrawing substituent, the effect being linearly correlated with the Hammett values (Figure 2.2.6). This is in agreement with the resonance structures in Figure 2.2.3.A.

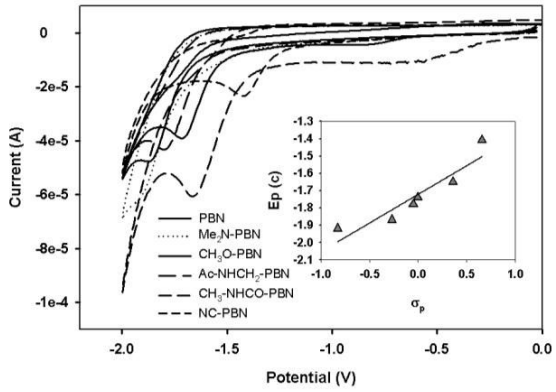


Figure 2.2.6. Reduction of PBN and compounds **1**-**6** in water containing 50 mM of NaCl at 0.1 $V.s^{-1}$. Insert is the correlation of Hammett Sigma Para Constants (σ_p) values with reductive potentials (R^2=0.87).

In a recent report we studied the electrochemical properties of *N-tert*-butyl substituted nitrone derivatives.[70] We observed that the polar effect was less pronounced on both reduction and oxidation potentials when compared to the data reported herein. This indicates the stronger polar effect of aromatic substituents compared with *N-tert*-butyl in agreement with the literature.[21, 23]

Table 2.2.1. Physical-chemical and electrochemical properties of PBN derivatives.

Compounds		σ_p [a]	**In CH_3CN**[b]			**In H_2O**[f]	**EPR**[i]
			E_p(c) (V)[c]		E_p(a) (V)[c]	E_p(c) (V)	k_{pN}/k_{TN} (± 0.05)
			1st peak	2nd peak			
4-Me_2N-PBN	**1**	-0.83	-2.30 ± 0.04	-2.49 ± 0.03	1.29 ± 0.05[e]	-1.92	0.16
4-MeO-PBN	**2**	-0.27	-2.15	-2.41 ± 0.09	1.42 ± 0.10	-1.87	0.25
4-AcNHCH$_2$-PBN	**3**	-0.05	-2.02	-2.17 ± 0.01	1.61 ± 0.06	-1.78	0.23
PBN	-	0	-2.02	-2.17 ± 0.01 (-2.40[d])	1.62 ± 0.06 (1.47[d])	-1.74 (-1.88[g])	0.13
4-MeNHCO-PBN	**4**	0.36	-1.82 ±0.11	-2.51 ± 0.13	1.79 ± 0.09	-1.65	n.d.[j]
4-HOOC-PBN	**5**	0.45	-2.18		1.82 ± 0.08	n.d.[h]	0.22
4-NC-PBN	**6**	0.66	-1.68 ±0.03	-2.37 ± 0.04	1.94 ± 0.04	-1.41	0.15

[a] Data from Hansch *et al*.[73] [b] Containing 50 mM of TBAP. [c]Average of three experiments. [d] Data from McIntire *et al*. in acetonitrile containing 0.10 M of TEAP *vs*. SCE at Pt with a sweep rate of 150 mV/s.[21] [e] Two supplementary peaks were observed at 0.84 ± 0.05 V and 1.67 ± 0.04 V. [f] Containing 50 mM of NaCl. [g] Data from McIntire *et al*. in water containing 0.10 M of $LiClO_4$ *vs*. SCE at Pt with a sweep rate of 150 mV/s.[21] [h]Not determined due to insolubility in water. [i] Ratio of the second-order rate constants for the phenyl radical trapping by various nitrones (k_{pN}) in benzene. [j] Not determined.

We next studied the spin-trapping properties of the derivatives using the phenyl radical ($Ph^{\bullet}$) adduct formation in benzene in the presence of 1,3,5-tri[(N-(1-diethylphosphono)-1-methylethyl) *N*-oxy-aldimine] benzene (TN) as competitive scavenger. [29] The $Ph^{\bullet}$ was generated by UV photolysis of a solution containing large excess of iodobenzene in the presence of TN and of the nitrone of interest, denoted as N. The $Ph^{\bullet}$ spin trapping rate was monitored by measuring the intensity (as the signal area) of the EPR signal of the corresponding adducts (Figure 2.2.6). The standard kinetic competition model employed as described elsewhere [29] yielded equation 1. In this equation, the second-order rate constants for $Ph^{\bullet}$ trapping by the nitrone N and TN are denoted as k_{pN} and k_{TN} respectively, while *r* and *R* represent the trapping rate by TN only in presence of N, and by both TN and N, respectively.

$$R/r = 1 + k_{pN}[N] / k_{TN}[TN] \quad (1)$$

By plotting the *R*/*r* ratio as a function of the [N]/[TN]ratio for each nitrone, a straight line was obtained with a slope equal to k_{pN}/k_{TN}. Five experiments were performed at five different [N]/[TN] ratios kept between 1 and 4.

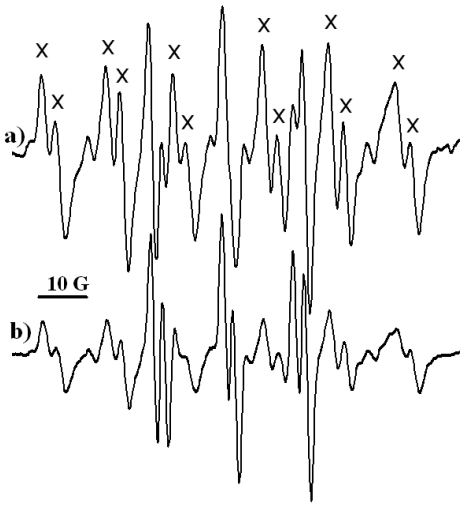


Figure 2.2.7. EPR signals recorded in benzene by photolysis of a 3 $mol.L^{-1}$ phenyl iodide solution in the presence of nitrone **6** and TN at two different concentration ratios [**6**]/[TN] : a) [**6**]/[TN] = 1.6 ([**6**] = 32 $mmol.L^{-1}$ and [TN] = 20 $mmol.L^{-1}$; b) [**6**]/[TN] = 3.2 ([**6**] = 32 $mmol.L^{-1}$ and [TN] = 10 $mmol.L^{-1}$. The peaks topped by a cross (x) correspond to the phenyl radical adduct of TN.

All the nitrones tested were found to be less efficient than TN with values of k_{pN}/k_{TN} ranging from 0.13 to 0.25 (Table 2.2.1). The PBN was the least potent of the series while MeOPBN (**2**) was the most potent in trapping $Ph^{\bullet}$. The absence of any correlation between the rate of trapping and the Hammett values is univocally observed in Figure 2.2.8.

The nature of radical addition to nitrone has been thoroughly studied over the past decades. It has been suggested that the positive ρ values are due to the nucleophilic character of the radicals as observed for alkyl,[32, 74] hydroxyl and phenyl radicals.[31] The substituent effect was also interpreted in terms of electron-transfer interaction between the free radical and the nitrone compound with a negative ρ value for phenyl radical bearing an EWG while those bearing an EDG led to a positive slope.[34] It was concluded that the reaction of the unsubstituted phenyl radical is an intermediate case. For the sake of comparison we added in Figure 2.2.8 the data from Murofushi et al.[34] and from Sueshi et al.[31] which further confirm the absence of any marked polar effect.

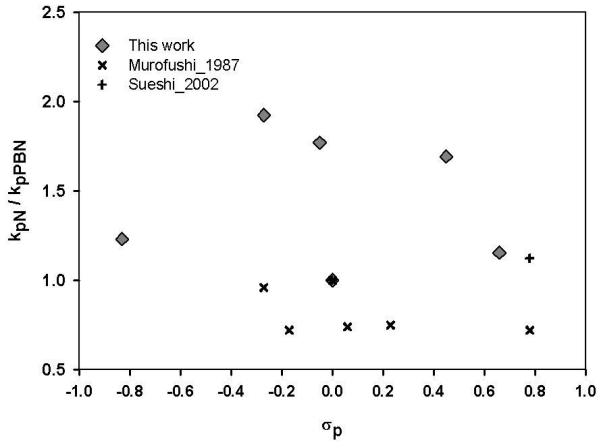


Figure 2.2.8. Plots of relative rate constant of phenyl radical trapping vs. Hammett sigma para constants (σ_p). (◇) This work. (×) Data from Murofushi et al.[34] (+) Data from Sueshi et al.[31]

Some inconstancies on the polar effect on the reactivity of nitrone have already been noted. The observed positive slope for HO• trapping by PBN derivatives, and the conclusion that HO• addition to nitrones is nucleophilic in nature[31] is contrary to the report by De

Vleeschouwer *et al*.[35] which demonstrated the electrophilic character of HO• using natural population analysis. This latter group also concluded to a moderate nucleophilic nature for •CH_3 that is in agreement with the conclusion of Sueishi et al.[32] According to the electrophilic scale of De Vleeschouwer *et al*., phenyl radical exhibits a moderate nucleophilic nature while our experimental data and those of other suggest the absence nucleophilic properties. This suggests that the sign of ρ value is more dependent on the nature of radical addition than on the intrinsic characteristic of the radical itself.

With regards to the rate of trapping by *N-tert*-butyl substituted nitrone, a reasonable correlation was observed with the nitronyl-C charge density suggesting a nucleophilic nature of phenyl radical.[70] With more compounds to add in the correlation (Figure 2.2.9) the nucleophilic nature of $Ph^{\bullet}$ addition appears less pronounced although a slight polar effect is still noticeable. This may also suggest that the reaction of $Ph^{\bullet}$ to nitrone is rather orbital-controlled than charge-controlled.

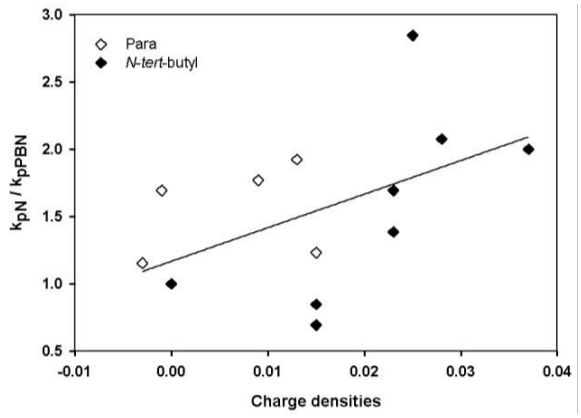


Figure 2.2.9. Correlation of the nitronyl carbon charge densities from Durand *et al*.[20] with experimental relative rate constants of phenyl addition to nitrones (k_{pN}/k_{pPBN}) (y=24.98x + 1.167; R =0.269) including *para*-substituted nitrones (marked as▲) from Rosselin *et al*.[70]

III. Conclusion

In summary, we reported the polar effect of *para*-substituents on the electrochemical properties of α-Phenyl-*N*-*tert*-butyl nitrones. Both in aqueous and organic solution, compounds bearing an electron-withdrawing group were more easily reduced than those having an electron-donating group, the effect being linearly correlated with the Hammett values of the substituent. An opposite trend was observed for the oxidation. The polar effect can be explained by the stabilization of the intermediate nitroxide formed through resonance structures. The ease of oxidation of nitrone derivatives has been found to correlate with antioxidant properties[62, 70] and therefore the lowest oxidation potential of 4-Me_2N-PBN and 4-MeO-PBN compared to PBN suggests they may exhibit improved antioxidant properties. The relative rate of Phenyl radical trapping showed no correlation with the Hammett values indicating the absence of a polar effect in agreement with previous data. This further supports the non nucleophilic nature of phenyl radical. Among the nitrones tested, 4-MeO-PBN exhibited interesting properties such as low oxidation potential, good trapping properties making the ether bond an efficient linker for *para*- functionalization of the α-phenyl N-tert-butyl nitrone.

IV. Experimental Section

General methods of cyclic voltammetry and spin-trapping kinetic experiments are described in part 1 of this chapter. The only difference in the spin-trapping kinetic experiments is the concentration of nitrones **1**-**6** which was varied from 5 to 35 mmol.L^{-1}.

Chapter II

Towards the improvement of the intrinsic properties of α-Phenyl-*N*-*tert*-butyl Nitrone

Chapter II – Part 3

α-Phenyl-*N*-cyclohexyl Nitrones: Preparation and Use as Spin-Traps

Part of this work will be submitted for publication as: Marie Rosselin, Kamal Zéamari, Fanny Choteau, Quentin Hericher, Béatrice Tuccio and Grégory Durand.

I. Introduction

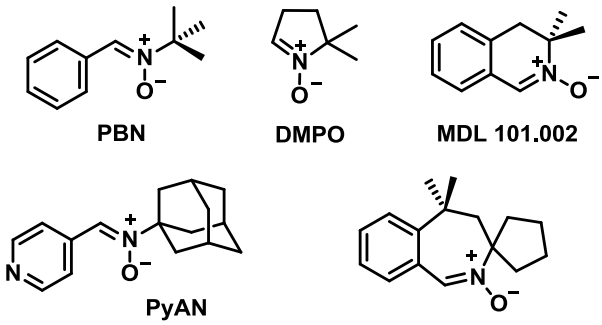
Nitrone-based compounds have been widely used as spin-traps for detecting transient free radicals where the nitronyl group reacts with a free radical to form a stable and identifiable aminoxyl radical that is detected by electron paramagnetic resonance (EPR) spectroscopy.[69, 75] Two families of nitrones are mainly employed to do so, the cyclic nitrones derived from the 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and the linear ones derived from the α-phenyl-*N*-*tert*-butyl nitrone (PBN). Aside from their use as spin-traps, the ability of nitrones to prevent oxidative stress-mediated damage in *in-vitro*, *in-vivo* and *ex-vivo* models has made them promising synthetic antioxidants with considerable potential as therapeutics.[4, 5, 59]

While the rate constant of free radicals addition to nitrones is rather high, ranging usually from 10^7 to 10^9 $M^{-1}s^{-1}$, in some cases, *i.e*. superoxide radical, the reaction is significantly slower rendering the detection difficult. It is therefore of the utmost importance to design nitrones that have the highest rate constant of free radical trapping when using them as probes. The ease of detection of a free radical is also determined by the stability of the aminoxyl spin-adduct. In general, cyclic nitrones lead to more stable spin adducts than linear ones and therefore, several analogues of DMPO with improved stability have been designed.[75] One can cite the phosphorylated analogue 5-diethoxyphosphoryl-5-methyl-1pyrroline *N*-oxide (DEPMPO), [76] the ester 5-ethoxycarbonyl-5-methyl-1pyrroline *N*-oxide (EMPO),[77] the 2-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO)[78] or an amido derivative (AMPO).[13, 36] On the other hand, efforts to synthesize analogs of PBN with improved adduct stability have met with limited success when compared to their cyclic analogs.[12, 79, 80] In our group, series of para- and *N*-*tert*-butylsubstituted PBN derivatives have been prepared but although some of our derivatives proved more potent than PBN, their rate of trapping remained within the same order of magnitude.[20, 70]

Several cyclic variants of PBN have been reported and examined as spin-traps and/or therapeutic agents. Thomas and colleagues reported the synthesis of 3,3-dimethyl-3,4-dihydroisoquinoline *N*-oxide (MDL 101,002, Figure 1) and several analogs whose presence of the cycle made the nitronyl group more reactive toward radical because of restricted rotation and higher accessibility.[60, 81] They also reported higher energy HOMO orbital and lower energy LUMO orbital for their lead compound compared with PBN making it more reactive toward both electrophilic and nucleophilic radicals.[60, 81] Hideg and colleagues also reported a series of cyclic variants of PBN derived from adamantane (PyAN, Figure 1) whose hydroxyl adduct stability was increased for the most efficient derivative by ~7 times compared to that of PBN.[82, 83] An heteroarylnitrone with a *N*-cyclohexyl group showed interesting antioxidant properties although surprisingly it failed to show detectable $HO^{\bullet}$ adduct signals while its analog with a *N-tert*-butyl group did.[84] With the rational that increasing the lipophilicity as a result of an expansion of the nitrone-containing ring may improve the activity, series of 2-Benzazepine nitrones were developed with the derivatives bearing a spirocyclic moiety (SpFigure 1) being significantly more potent than PBN in protecting neuronal cells against oxidative stress.[85] A DMPO derivative with a rigid spirolactonyl moiety was prepared and showed higher rate constant of superoxide trapping compared to several DMPO analogs.[86]

In connection with our program devoted to the design of targeted nitrone-based spin-traps with improved reactivity towards free radicals we report herein the design of bifunctional α-phenyl-*N*-cyclohexyl-nitrones (Figure 2.3.1). Our expectation is that the cyclohexyl ring will impart lipophilicty to the molecule, high reactivity of the nitronyl group and, stability of the spin adduct formed. Further functionalization of the nitrone to accommodate target-specific groups may achieve high reactivity to radicals, longer adduct half-life, and enhanced and controlled bioavailability in a unified molecular design (Figure 2.3.1). The synthesis of the α-

phenyl-*N*-cyclohexyl-nitrone **4** and its OtBu-protected form **3** is reported. The water solubility, lipophilicity and electrochemical properties of these two derivatives were determined. Their ability to trap oxygen- and carbon-centered radicals were studied using EPR experiments. Moreover, EPR competition kinetic experiments are currently under progress to determine the relative rate constants of spin-adduct formation.



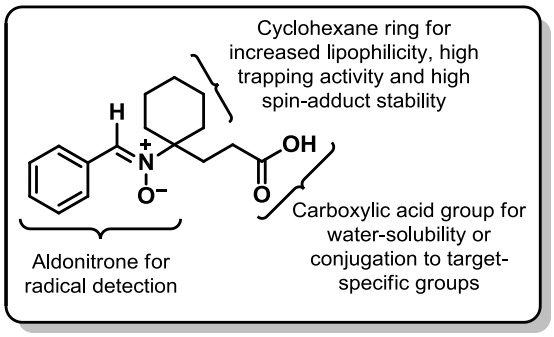
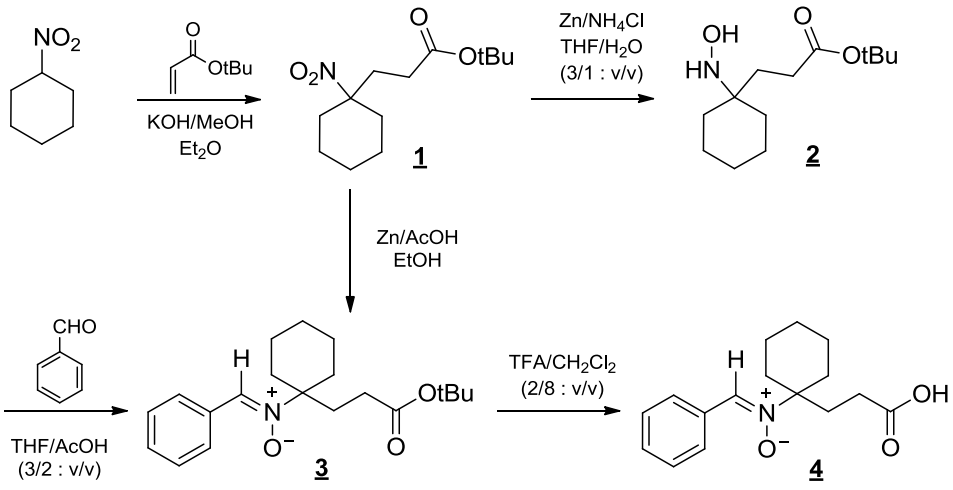


Figure 2.3.1. Structure of PBN, DMPO, examples of structure of cyclic variants of phenyl- and arylnitrones, and general concept of α-Phenyl-*N*-cyclohexyl nitrone.

II. Results and Discussion

The synthesis of nitrone **4** is summarized in Scheme 1. Nitrocyclohexane was used as starting material and the first step consisted in the grafting of *tert*-butyl acrylate onto the cyclohexyl ring through a Michael reaction. Several experimental conditions were tested and Table 2.3.1 summarizes the outcome of our screening. In our hands, the use of a 1/35 mixture of KOH/MeOH in dry Et_2O following the procedure described by Kolter *et al*. was the most efficient condition,[87] leading to compound **1** in 67 % yield after flash chromatography purification. When using *t*BuOK as a base, higher amount of *tert*-butyl acrylate were needed

to ensure a reasonable yield. Moreover degradation of the reaction mixture could occur in some cases with no compound **1** formed. Next step consisted in the condensation of the nitronyl group onto the bezaldehyde to form the nitronyl group. We investigated two synthetic strategies. The first synthetic route relied on the reduction of the nitro group to its hydroxylamine form which was further purified and isolated. Reduction was conducted in the presence of zinc dust and NH_4Cl in a THF/H_2O mixture (3:1, v/v) under argon atmosphere following our classical procedure.[9] Hydroxylamine **2** was obtained in 75% yield and was next condensed to benzaldehyde under argon atmosphere in a dry 3:2 THF/AcOH mixture (v/v) in the presence of molecular sieves to give nitrone **3** in 85% yield. The second synthetic route consisted in a one-pot reduction-condensation of the nitro derivative **1** onto the benzaldehyde in the presence of Zinc dust and AcOH in EtOH.[17] Under these conditions, nitrone **3** was isolated after purification in 30% yield and although this second strategy was one step shorter, it failed to improve the overall yield. Finally, removal of the *tert*-butoxy protecting group under acidic condition led to nitrone **4** in 92% yield. It is worth noting that despite the use of TFA no degradation of the nitronyl group was observed. Before any physical-chemical investigation both nitrone **3** and **4** were recrystallized twice in order to ensure high purity.



Scheme 2.3.1. Synthesis of α-Phenyl-*N*-cyclohexyl Nitrone derivatives.

Table 2.3.1. Optimization of Michael reaction to furnish compound **1**.

Solvent	Base	Reactant	Reaction Time	Temp.	Yield
THF	1.1 eq *t*BuOK	1.1 eq acrylate	Overnight	RT	31
THF	3 eq. *t*BuOK	1 eq. acrylate	Overnight	RT	28
THF	1.5 eq. *t*BuOK	1.5 eq. acrylate	Overnight	RT	40
Et_2O	KOH/MeOH 1/35	1 eq. acrylate	Overnight	0°C to RT	67

The water solubility of nitrones **3**, **4** and its sodium salt was determined using a UV spectroscopic method[70] and was compared to that of PBN (Table **2.3.2**). Nitrone **3** was found insoluble in water while nitrone **4** exhibited a water solubility of 2.1 g/L, which is ten times lower than that of PBN (21.4 g/L). Water solubility of nitrone **4** was significantly improved after conversion under carboxylic salt form (17.1 g/L). The relative lipophilicity (log k'_w) of the nitrones was measured by HPLC and values are reported in Table **2.3.2**. This confirms the higher lipophilic character of the ester nitrone **3** compared to nitrone **4** with log k'_w values of 3.56 and 2.32, respectively, whereas 1.68 was found for PBN in agreement with previous reports.[9] Calculated partition coefficients (Clog*P*) were also determined using Marvin software and a good correlation was observed between the experimental and the calculated data (R^2>0.999).

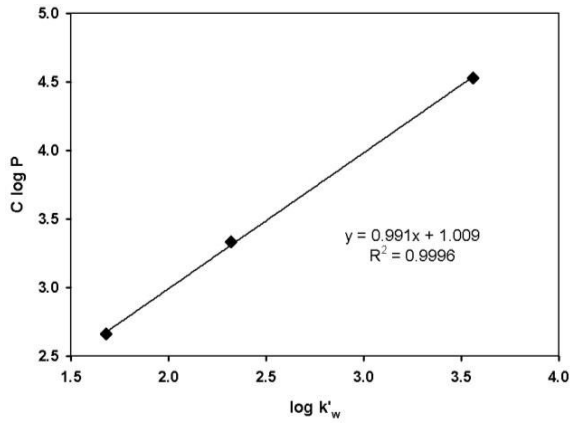


Figure 2.3.2. Partition coefficients (log k'_w) versus Clog P.

Table 2.3.2. Physical-chemical and electrochemical properties of nitrones **3** and **4**.

Nitrones	Water solubility (g/L)	Lipophilicity		E_p(c) (V)		E_p(a) (V)
		log *k*'w[c]	Clog *P*[d]	In H_2O[d]	In CH_3CN[e]	
3	-[a]	3.56	4.53	-[a]	-2.19; -2.07	1.56
4	2.1 (17.1)[b]	2.32	3.33	-1.90	-2.28 ; -2.12	1.62
PBN	21.4	1.68	2.66	-1.74	-2.23; -2.04	1.61

[a]Not soluble. [b]Carboxylate form. [c]Partition coefficient values obtained by HPLC. [d]Calculated octanol/water partition coefficient values obtained using Marvin software (http://www.chemaxon.com/marvin/help/index.html). [d]Containing 50 mM of NaCl. [e]Containing 50 mM of TBAP.

The electrochemical behavior of the two nitrones was investigated using cyclic voltammetry and values are reported in Table 2.3.2. We first carried out cyclic voltammetry in 50 mM NaCl aqueous solution. As already observed for other nitrones, the oxidation of the nitronyl group was not detected.[21, 22] On the contrary, we observed that nitrone **4** exhibited an irreversible one-step reduction, as shown in Figure 2.3.3, with a cathodic peak potential of -1.90 V vs. Ag/AgCl, that of the PBN being -1.74 V. Due to its insolubility in water, nitrone **3** was not tested. We next studied the electrochemical properties of the nitrones in acetonitrile containing tetra-butylammonium perchlorate (TBAP) as electrolyte. Previous works showed that PBN undergoes an irreversible one-electron oxidation and a one-step, two-electron reduction.[21, 24, 25] Compared to the aqueous conditions, oxidation of nitrone **3** and **4** was clearly observed in acetonitrile as shown in Figure 2.3.3 with values of 1.56 V and 1.62 V, respectively. Reduction of the nitrones in a non-aqueous medium showed the presence of two reduction potentials for the two nitrones **3** and **4** with ~0.15 V between each peak. A similar observation has been made for para-substituted-α-phenyl-*N*-*tert* butyl nitrone derivatives (see chapter 2, part 2). Only a modest ease of reduction was observed for nitrone **3** compared to nitrone **4** and PBN. This data shows that the presence of the cyclohexyl ring does not affect both reduction and oxidation potentials of the nitronyl group which is in agreement with

findings by Zuman and Exner,[23] McIntire et al.,[21] and our group on the effect of N-alkyl modification of phenyl nitrones.

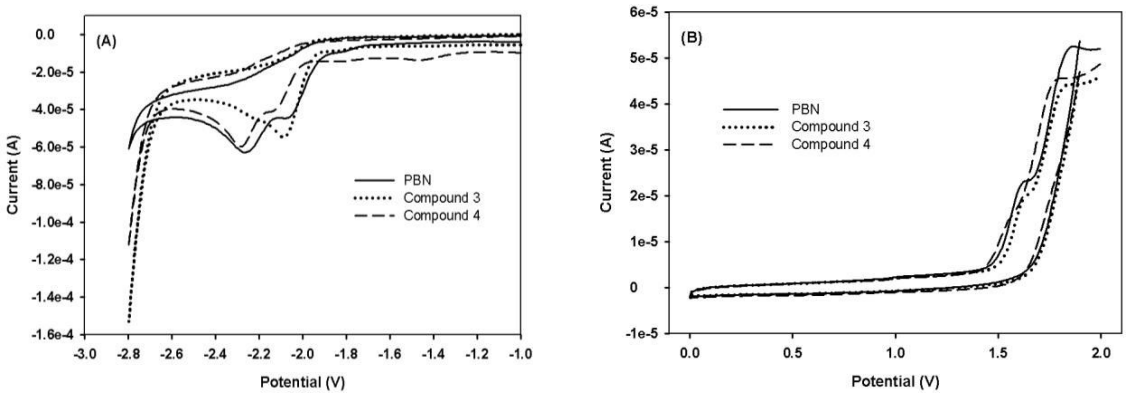


Figure 2.3.3. Cyclic voltammograms of PBN and compounds **3** and **4** in acetonitrile containing 50 mM of TBAP with a sweep rate of 0.1 $V.s^{-1}$; (A) reduction and (B) oxidation.

Spin trapping. The spin trapping ability of the two α-Phenyl-*N*-cyclohexyl PBN derivatives were evaluated by formation of oxygen-centered radical spin-adducts *i.e*. $MeO^{\bullet}$, $tBuO^{\bullet}$, $HOO^{\bullet}$, $O_2^{\bullet-}$ and $HO^{\bullet}$ adducts. The corresponding hyperfine coupling constants (hfcc's) are reported in Table 2.3.3. In most cases, the nitrones tested gave rise to a standard six-line EPR spectrum with more or less well-defined signals but whose values are in agreement with the literature.[19] The methoxy radical was generated by addition of lead tetraacetate ($PbOAc_4$) in a solution of DMSO/MeOH (9:1, v/v) containing the nitrone. Compound **3** and **4** were found to efficiently trap $MeO^{\bullet}$ radical with hfcc's in good agreement with PBN methoxy spin-adducts. The ill-defined spectrum obtained with compound **3** led to less precise determination of hfcc's, compared to compound **4** (Figure 2.3.4). The two compounds were found to trap $tBuO^{\bullet}$ radical, generated by UV-irradiation in DMSO solution containing $(tBuO)_2$, however, signals were broad. The hydroperoxyl radical was generated using hydrogen peroxide in pyridine solution. In this system, it's believed that the hydroperoxide ion reacts first by nucleophilic addition onto the nitronyl function, then, oxidation of the resulting hydroxylamine gives the hydroperoxyl radical spin-adduct. In our hand we observed broad

signals and despite bubbling of the solution with argon to remove the oxygen, no improvement of the line width was observed. EPR signals of the superoxide radical spin-adduct ($O_2^{\bullet -}$), generated using a solution of KO_2 in DMSO, were too weak to be analyzed. Concerning the hydroxyl radical generated in aqueous solution by hydrogen peroxide photolysis, compound **4** was not studied due to its insolubility in water. Unfortunately, only weak signals were observed for compound **3**, which can be explain by the instability of hydroxyl spin-adduct generally observed with PBN-type nitrones. We also investigated the trapping of a carbon-centered radical. The methyl radical was obtained by a Fenton reaction in DMSO/phosphate buffer solution (7:3, v/v) in presence of hydrogen peroxide. The two nitrones tested trapped $CH_3^{\bullet}$, leading to ill-defined signals but simulations gave a_N and a_H values in agreement with the methyl radical adduct of a PBN-type nitrone.

Table 2.3.3. EPR hyperfine coupling constants of different radical adducts of compounds **3** and **4**.

Compounds	**N-Cy-Hex-OH**		**N-Cy-Hex-OtBu**	
Radicals trapped	a_N	a_H	a_N	a_H
$CH_3O^{\bullet}$	14.4 G	2.9 G	13.7 G	2.3 G
$tBuO^{\bullet}$	13.7 G	2.3 G	13.7 G	2.2 G
$HOO^{\bullet}$	13.3 G	1.4 G	13.7 G	2.0 G
$CH_3^{\bullet}$	14.3 G	2.6 G	14.3 G	2.6 G
$OO^{-\bullet}$	Weak signals			
$HO^{\bullet}$	Weak signals		Not soluble	

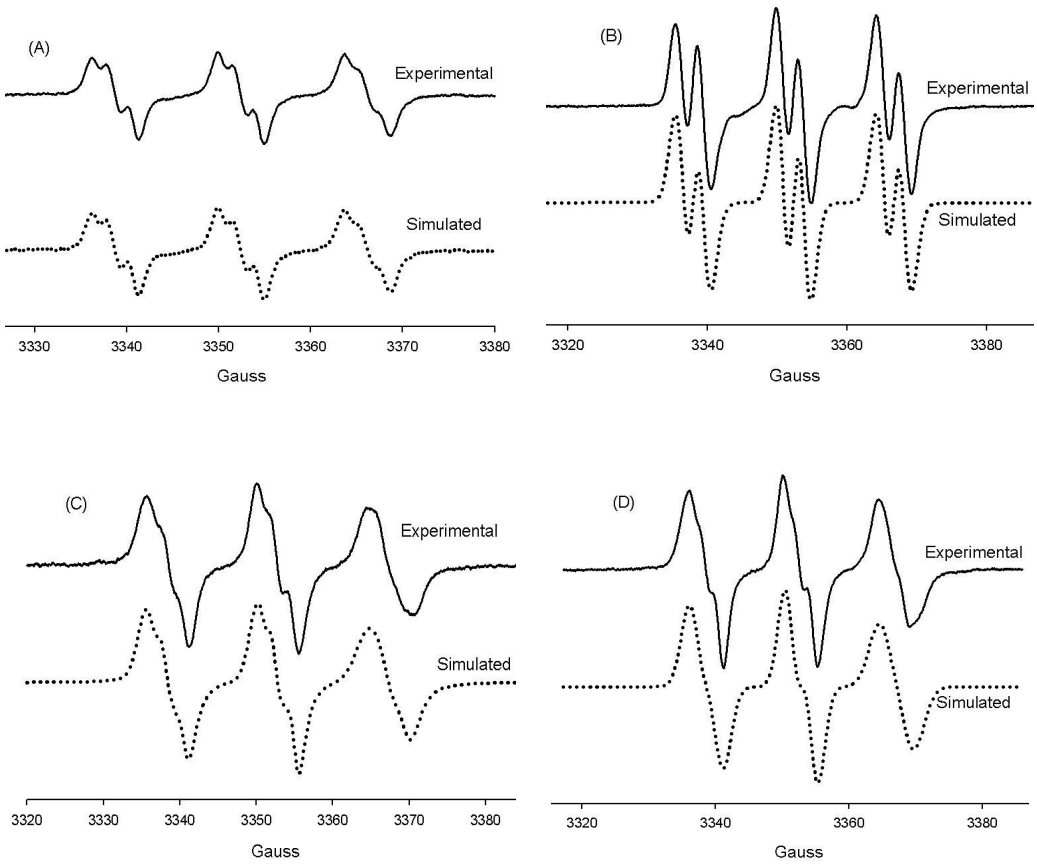


Figure 2.3.4. Methoxy radical spin-adduct of compound **3** (A) and compound **4** (B); Methyl radical spin-adduct of compound **3** (C) and compound **4** (D).

III. Conclusion

We have designed two α-phenyl-*N*-cyclohexyl-nitrones by addition of a rigid cyclohexyl ring instead of the *tert*-butyl group in order to improve the reactivity towards free radicals. The higher lipophilicity of the two derivatives compare to PBN was confirmed by HPLC. However, only the carboxylic salt form of compound **3** showed sufficient water solubility, making it suitable for biological evaluation. The redox properties were investigated using cyclic voltammetry and results have shown that the presence of a cyclohexyl ring had no influence on both oxidation and reduction potentials of the nitronyl group. This is not surprisingly as the substituent exerts no electronic effect onto the nitronyl function. The ability of the two compounds to trap oxygen- and carbon-centered radicals was next

confirmed by EPR experiments and kinetic experiments are currently in progress to evaluate if the cyclohexyl ring effectively stabilize the spin adduct formed. If interesting results are obtained, the next step will consist in the design of α-phenyl-*N*-cyclohexyl-nitrone derivatives with substituents in para position of the phenyl ring. Moreover, the carboxylic acid group will be used to graft specific-targeted ligands such as cell penetrating peptides.

IV. Experimental Section

General methods and materials for the synthesis, water solubility determination, log k'_W determination, Clog*P* values determination, spin-trapping experiments and cyclic voltammetry experiments are described in part 1 of this chapter.

Compound 1. Under argon atmosphere 5 g (38.7 mmol. 1 eq) of nitrocyclohexane were dissolved in Et_2O (8 mL). The solution was cooled down and 0.44 mL of a 1.5:8.5 KOH/MeOH solution (m/m) (1.11 mmol. 1/35 eq) and 4.9 mL of *tert*-butoxy acrylate (38.7mmol. 1 eq) were successively added dropwise. The solution was stirred at RT for 16 hours then AcOH was added until the pH of the solution reached ~5. The solvents were removed under vacuum and the resulting crude residue was purified by flash chromatography (cyclohexane/Et_2O 95:5 v/v) to lead to compound **1** (6.24 g, 25.93 mmol, 67%) as a yellow oil. R_f (cyclohexane/Et_2O) = 0.37. RMN ^1H ($CDCl_3$. 400 MHz) δ 2.46-2.41 (2H. m). 2.22-2.14 (4H. m). 1.64-1.34 (8H. m). 1.46 (3H. s). RMN ^{13}C ($CDCl_3$. 100 MHz) δ 171.4. 91.3. 80.6. 35.0. 33.4. 29.5. 28.1. 24.5. 22.4. MS (ESI+. *m/z*) : 258 [$(M+H)^+$]. 275 [$(M+NH_4)^+$]. 280 [$(M+Na)^+$]. 296 [$(M+K)^+$].

Compound 2. Under argon atmosphere. 1 g of compound **1** ($4.14.10^{-3}$ mol. 1 eq) and 0.33 g of NH_4Cl ($6.21.10^{-3}$ mol. 1.5 eq) were dissolved in 3:1 THF/H_2O mixture (v/v). The solution was cooled down and 1.07 g of Zinc dust (16.56 mmol. 4 eq) were added portion wise keeping the temperature below 15°C during the addition then the solution was stirred for two hours at RT and filtered off through a pad of celite. The solvents were removed under vacuum and the crude residue was purified by flash chromatography (cyclohexane/EtOAc. 8:2 v/v) to lead to

compound **2** (0.75 g, 3.09 mmol, 75%) as a white powder. R_f (cyclohexane/EtOAc. 8:2 v/v) = 0.22. ^{1}H NMR ($CDCl_3$. 400 MHz) δ 5.55 (1H. bs). 2.19 (2H. t. *J*= 6.0 Hz). 1.71 (2H. t. *J*= 6.0 Hz). 1.60-1.10 (10H. m). 1.39 (9H. s). ^{13}C NMR ($CDCl_3$. 100 MHz) δ 174.4. 80.2. 57.82. 32.5. 31.2. 29.4. 28.0. 25.9. 21.8. MS (ESI+. *m/z*) : 244 [$(M+H)^+$]. 266 [$(M+Na)^+$]. 282 [$(M+K)^+$].

Compound 3. Under argon atmosphere. 0.21 g of benzaldehyde (2.03 mmol. 1 eq) and 0.37 g of compound **2** (1.62 mmol. 0.8 eq) were dissolved in a 3:2 THF/AcOH mixture (v/v) in the presence of 4 Å molecular sieves. The solution was stirred at 60°C for 16 hours and 0.2 eq of compound **2** was added after 3 and 14 hours of stirring. The crude mixture was filtered off through a pad of celite and the solvents were removed under vacuum. The resulting crude residue was purified by flash chromatography (cyclohexane/EtOAc. 8:2 v/v) to lead to compound **3** (0.57 g. 1.73 mmol. 85%) as a white powder. R_f (cyclohexane/EtOAc. 8:2 v/v) = 0.40. ^{1}H NMR ($CDCl_3$. 400 MHz) δ 8.29 (2H. m). 7.50-7.35 (4H. m). 2.30-2.10 (6H. m). 1.84 (2H. m). 1.61 (4H. m). 1.50 (2H. m). 1.40 (9H. s). ^{13}C NMR ($CDCl_3$. 100 MHz) δ 172.4. 131.7. 130.8. 130.2. 128.9. 128.5. 80.5. 75.2. 34.3. 29.6. 28.0. 25.4. 22.30. MS (ESI+. *m/z*) : 332 [$(M+H)^+$]. 354 [$(M+Na)^+$]. 370 [$(M+K)^+$]. HR-MS (ESI+.m/z) calcd for $C_{20}H_{29}O_3N$ [(M+H)+] : 332.2226. found 332.2252.

Compound 4. Under argon atmosphere. 0.5 g of compound **3** (1.51 mmol) were dissolved in 2:8 TFA/CH_2Cl_2 mixture (v/v). The solution was stirred for 4 hours at RT then the solvents were removed under vacuum. The resulting crude mixture was purified by flash chromatography (EtOAc/cyclohexane. 6:4 v/v) to lead to compound **4** (0.38 g. 1.39 mmol. 92%) as a white powder. R_f (cyclohexane/EtOAc. 6:4 v/v) = 0.12. ^{1}H NMR ($CDCl_3$. 400 MHz) δ 8.29 (2H. m). 7.53 (1H. s). 7.44 (3H. m). 2.32-2.28 (2H. m). 2.21-2.17 (4H. m). 1.90-1.83 (2H. m). 1.63-1.58 (4H. m). 1.50 (2H. m). ^{13}C NMR (C DCl_3. 100 MHz) δ 176.6. 134.9. 131.3. 130.1. 129.8. 128.7. 75.5. 34.4. 32.6. 28.5. 25.5. 22.3. MS (ESI+. *m/z*) : 276 [$(M+H)^+$].

298 [$(M+NH_4)^+$]. 314 [$(M+K)^+$]. HR-MS (ESI+.m/z) calcd for $C_{16}H_{21}O_3N$ [(M+H)+] : 276.1600. found 276.1611.

Determination of water solubility. For PBN and nitrones **3** and **4**, a UV-calibration curve at 290 nm was established from solutions ranging from 10^{-3} to 10^{-2} g/L ($R > 0.997$). A saturated solution of nitrone was prepared at 40°C and then let stand at RT overnight. After centrifugation (12000 g – 15 minutes) at room temperature, the concentration of the supernatant solution was determined using the calibration curve.

Determination of log k'_W values. Compounds were dissolved in MeOH at 0.5 mg/mL and were injected onto a C18 reverse phase column (250 mm x 4.6 mm. 5µm). The compounds were eluted at various MeOH and water ratios (9:1 to 4:6 v/v) with 0.1% acetic acid using a flow rate of 0.8 mL/min. The column temperature was 25°C. and the UV detector wavelength was $\lambda = 298$ nm. Linear regression analysis were performed on four data points for compound **3** (from 9:1 to 6:4; $R^2 > 0.997$); compound **4** (from 7:3 to 4:6; $R^2 > 0.999$) and PBN (from 7:3 to 4:6; $R^2 > 0.999$). The log *k*' values were calculated by using the equation: $\log k' = \log((t-t_0)/t_0)$. where *t* is the retention time of the nitrone and t_0 is the elution time of MeOH. which is not retained on the column.

Determination of Clog*P* values. The partition coefficient octanol/water (Clog*P*) was determined using MarvinSketch 5.9.0 that is available at www.chemaxon.com/marvin.

Cyclic Voltammetric Measurement. The electrochemical experiments were carried out using a three-electrode cell in a dry argon atmosphere at room temperature. An Ag/AgCl/saturated NaCl electrode was used as the reference electrode and a platinum wire as the auxiliary electrode. The working electrode (glassy carbon) was polished prior to each experiment using a 0.04 µm aqueous alumina slurry on a wetted polishing cloth.

References

[1] F.A. Villamena, J.L. Zweier, Detection of reactive oxygen and nitrogen species by EPR spin trapping, Antioxid. Redox Signaling, 6 (2004) 619-629.

[2] A.E. Dikalova, M.B. Kadiiska, R.P. Mason, An in vivo ESR spin-trapping study: free radical generation in rats from formate intoxication--role of the Fenton reaction., Proc. Natl. Acad. Sci. USA, 98 (2001) 13549-13553.

[3] M.B. Kadiiska, M.J. Burkitt, Q.H. Xiang, R.P. Mason, Iron supplementation generates hydroxyl radical in vivo. An ESR spin-trapping investigation., J. Clin. Invest., 96 (1995) 1653-1657.

[4] R.A. Floyd, R.D. Kopke, C.-H. Choi, S.B. Foster, S. Doblas, R. Towner, A., Nitrones as therapeutics, Free Radical Biol. Med., 45 (2008) 1361-1374.

[5] F.A. Villamena, A. Das, K.M. Nash, Potential implication of the chemical properties and bioactivity of nitrone spin traps for therapeutics, Future Medicinal Chemistry, 4 (2012) 1171-1207.

[6] K.R. Maples, A.R. Green, R.A. Floyd, Nitrone-related therapeutics: potential of NXY-059 for the treatment of acute ischaemic stroke, CNS Drugs, 18 (2004) 1071-1084.

[7] M. Hardy, A. Rockenbauer, J. Vasquez-Vivar, C. Felix, M. Lopez, S. Srinivasan, N. Avadhani, P. Tordo, B. Kalyanaraman, Detection, characterization, and decay kinetics of ROS and thiyl adducts of mito-DEPMPO spin trap, Chem. Res. Toxicol., 20 (2007) 1053-1060.

[8] M.P. Murphy, K.S. Echtay, F.H. Blaikie, J. Asin-Cayuela, H.M. Cocheme, K. Green, J.A. Buckingham, E.R. Taylor, F. Hurrell, G. Hughes, S. Miwa, C.E. Cooper, D.A. Svistunenko, R.A.J. Smith, M.D. Brand, Superoxide Activates Uncoupling Proteins by Generating Carbon-centered Radicals and Initiating Lipid Peroxidation: Studies using a Mitochondria-Targeted Spin Trap derived from alpha -phenyl-N-tert-butylnitrone, J. Biol. Chem., 278 (2003) 48534-48545.

[9] G. Durand, B. Poeggeler, S. Ortial, A. Polidori, F.A. Villamena, J. Böker, R. Hardeland, M.A. Pappolla, B. Pucci, Amphiphilic amide nitrones: A new class of protective agents acting as modifiers of mitochondrial metabolism, J. Med. Chem., 53 (2010) 4849-4861.

[10] S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M.A. Pappolla, J. Boeker, R. Hardeland, B. Pucci, Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents, J. Med. Chem., 49 (2006) 2812-2820.

[11] A. Zeghdaoui, B. Tuccio, J.-P. Finet, V. Cerri, P. Tordo, beta -Phosphorylated alpha -phenyl-N-tert-butylnitrone (PBN) analogs: a new series of spin traps for oxyl radicals, J. Chem. Soc., Perkin Trans. 2 (1995) 2087-2089.

[12] A. Allouch, V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, Preparation and use as spin trapping agents of new ester-nitrones, Org. Biomol. Chem., 1 (2003) 593-598.

[13] F.A. Villamena, S. Xia, J.K. Merle, R. Lauricella, B. Tuccio, C.M. Hadad, J.L. Zweier, Reactivity of superoxide radical anion with cyclic nitrones: Role of intramolecular H-Bond and electrostatic effects, J. Am. Chem. Soc, 129 (2007) 8177-8191.

[14] S.-U. Kim, F.A. Villamena, Reactivities of Superoxide and Hydroperoxyl Radicals with Disubstituted Cyclic Nitrones: A DFT Study, J. Phys. Chem. A, 116 (2011) 886-898.

[15] H. Karoui, C. Nsanzumuhire, F. Le Moigne, M. Hardy, D. Siri, E. Derat, A. Rockenbauer, O. Ouari, P. Tordo, Synthesis and Spin-Trapping Properties of a Trifluoromethyl Analogue of DMPO: 5-Methyl-5-trifluoromethyl-1-pyrroline N-Oxide (5-TFDMPO), Chemistry – A European Journal, 20 (2014) 4064-4071.

[16] E.G. Janzen, R.C. Zawalski, Synthesis of nitronyl alcohols and their benzoate esters, J. Org. Chem., 43 (1978) 1900-1903.

[17] F. Choteau, B. Tuccio, F.A. Villamena, L. Charles, B. Pucci, G. Durand, Synthesis of Tris-hydroxymethyl-Based Nitrone Derivatives with Highly Reactive Nitronyl Carbon, J. Org. Chem., 77 (2012) 938-948.

[18] L.M.L. Nollet, L.S.P.D. Gelder, Handbook of water analysis, CRC Press, 2000.

[19] G. Buettner, Spin Trapping: ESR parameters of spin adducts, Free Radic. Biol. Med., 3 (1987) 259-303.

[20] G. Durand, F. Choteau, B. Pucci, F.A. Villamena, Reactivity of Superoxide Radical Anion and Hydroperoxyl Radical with α-Phenyl-N-tert-butylnitrone (PBN) Derivatives, J. Phys. Chem. A, 112 (2008) 12498-12509.

[21] G.L. McIntire, H.N. Blount, H.J. Stronks, R.V. Shetty, E.G. Janzen, Spin Trapping in Electrochemistry. 2. Aqueous and Nonaqueous Electrochemical Characterization of Spin Traps, J. Phys. Chem., 84 (1980) 916-921.

[22] B. Tuccio, R. Lauricella, C. Frejaville, J.-C. Bouteiller, P. Tordo, Decay of the hydroperoxyl spin adduct of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: an EPR kinetic study, J. Chem. Soc., Perkin Trans. 2, (1995) 295-298.

[23] P. Zuman, O. Exner, Collect. Czech. Chem. Comm., 30 (1965) 1832-1852.

[24] B. Tuccio, P. Bianco, J.-C. Bouteiller, P. Tordo, Electrochemical characterisation of β-phosphorylated nitrone spin traps, Electrochimica Acta, 44 (1999) 4631-4634.

[25] B.J. Acken, D.E. Gallis, J.A. Warshaw, D.R. Crist, Electrochemical behavior of C-methoxy, amino, cyano, and mercapto nitrones, Canadian J. Chem., 70 (1992) 2076-2080.

[26] K. Nakahara, S. Iwasa, J. Iriyama, Y. Morioka, M. Suguro, M. Satoh, E.J. Cairns, Electrochemical and spectroscopic measurements for stable nitroxyl radicals, Electrochimica Acta, 52 (2006) 921-927.

[27] V. Suryanarayanan, S. Yoshihara, T. Shirakashi, Electrochemical behavior of carbon electrodes in organic liquid electrolytes containing tetrafluoroborate and hexafluorophosphate anionic species in different non-aqueous solvent systems, Electrochimica Acta, 51 (2005) 991-999.

[28] F.A. Villamena, M.H. Dickman, D.R. Crist, Nitrones as Ligands in Complexes of Cu(II), Mn(II), Co(II), Ni(II), Fe(II), and Fe(III) with N-tert-Butyl-α-(2-pyridyl)nitrone and 2,5,5-Trimethyl-1-pyrroline-N-oxide†, Inorganic Chemistry, 37 (1998) 1446-1453.

[29] V. Roubaud, H. Dozol, C. Rizzi, R. Lauricella, J.-C. Bouteiller, B. Tuccio, J. Chem. Soc., Perkin Trans. 2, (2002) 958-964.

[30] R. Lauricella, J.-C. Bouteiller, B. Tuccio, Phys . Chem. Chem. Ph ys., 7 (2005) 399–404.

[31] Y. Sueishi, C. Yoshioka, C. Olea-Azar, L.A. Reinke, Y. Kotake, Substituent effect on the rate of the hydroxyl and phenyl radical spin trapping with nitrones, Bull. Chem. Soc. Jpn., 75 (2002) 2043-2047.

[32] Y. Sueishi, D. Yoshioka, C. Yoshioka, S. Yamamoto, Y. Kotake, High static pressure alters spin trapping rates in solution. Dependence on the structure of nitrone spin traps, Org. Biomol. Chem., 4 (2006) 896-901.

[33] Y. Abe, S. Seno, K. Sakakibara, M. Hirota, Spin trapping of oxygen-centered radicals by substituted N-benzylidene-tert-butylamine N-oxides, J. Chem. Soc., Perkin Trans. 2, (1991) 897-903.

[34] K. Murofushi, K. Abe, M. Hirota, Substituent effect on the spin-trapping reactions of substituted N-benzylidene-tert-butylamine N-oxides, J. Chem. Soc., Perkin Trans. 2, (1987) 1829-1833.

[35] F. DeVleeschouwer, V. VanSpeybroeck, M. Waroquier, P. Geerlings, F. DeProft, Electrophilicity and Nucleophilicity Index for Radicals, Org. Lett., 9 (2007) 2721-2724.

[36] F.A. Villamena, A. Rockenbauer, J. Gallucci, M. Velayutham, C.M. Hadad, J.L. Zweier, Spin trapping by 5-carbamoyl-5-methyl-1-pyrroline N-oxide (AMPO): Theoretical and experimental studies, J. Org. Chem., 69 (2004) 7994-8004.

[37] F.A. Villamena, J.K. Merle, C.M. Hadad, J.L. Zweier, Rate Constants of Hydroperoxyl Radical Addition to Cyclic Nitrones: A DFT Study, J. Phys. Chem. A 111 (2007) 9995-10001.

[38] G. Durand, A. Polidori, O. Ouari, P. Tordo, V. Geromel, P. Rustin, B. Pucci, Synthesis and preliminary biological evaluations of ionic and nonionic amphiphilic alpha-phenyl-N-tert-butylnitrone derivatives, J. Med. Chem., 46 (2003) 5230-5237.

[39] D.R. Duling, Simulation of multiple isotropic spin-trap EPR spectra, J. Magn. Reson., Ser. B 104 (1994) 105-110.

[40] J.W. Labanowski, J. Andzelm, Density Functional Methods in Chemistry, Springer, New York, 1991.

[41] R.G. Parr, W. Yang, Density Functional Theory in Atoms and Molecules, New York, 1989.

[42] A.D. Becke, Density-functional exchange-energy approximation with correct asymptotic behavior, Phys. Rev. , 38 (1988) 3098-3100.

[43] A.D. Becke, A new mixing of Hartree-Fock and local-density-functional theories, J. Chem. Phys., 98 (1993) 1372-1377.

[44] W.J. Hehre, L. Radom, P.V. Schleyer, J.A. Pople, Ab Initio Molecular Orbital Theory, New York, 1986.

[45] V. Barone, M. Cossi, J. Tomasi, A new definition of cavities for the computation of solvation free energies by the polarizable continuum model, J. Chem. Phys., 107 (1997) 3210–3221.

[46] V. Barone, M. Cossi, J. Tomasi, Geometry optimization of molecular structures in solution by the polarizable continuum model, J. Comput. Chem., 19 (1998) 404.

[47] M. Cossi, V. Barone, R. Cammi, J. Tomasi, Ab initio study of solvated molecules: A new implementation of the polarizable continuum model, Chem. Phys. Lett., 255 (1996) 327-335.

[48] J. Tomasi, B. Mennucci, R. Cammi, Quantum mechanical continuum solvation models, Chem. Rev., 105 (2005) 2999–3093.

[49] J. Tomasi, M. Persico, Molecular interactions in solution: an overview of methods based on continuous distributions of the solvent, Chem. Rev., 94 (1994) 2027–2094.

[50] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery, Jr., T. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G.A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, J.A. Pople, Gaussian 03, in, Gaussian, Inc., Pittsburgh PA, 2003.

[51] C. Lee, W. Yang, R.G. Parr, Phys. Rev. B, 37 (1988) 785-789.

[52] A.E. Reed, L.A. Curtiss, F.A. Weinhold, NPA, Chem. Rev., 88 (1988) 899-926.

[53] J.P. Foster, J. Weinhold, J. Am. Chem. Soc, 102 (1980) 7211-7218.

[54] A.P. Scott, L. Radom, Harmonic vibrational frequencies: An evaluation of hartree-fock, moeller-plesset, quadratic configuration interaction, density functional theory, and semiempirical scale factors, J. Phys. Chem., 100 (1996) 16502-16513.

[55] R. Lauricella, A. Allouch, V. Roubaud, J.-C. Bouteiller, B. Tuccio, A new kinetic approach to the evaluation of rate constants for the spin trapping of superoxide/hydroperoxyl radical by nitrones in aqueous media, Org. Biomol. Chem., 2 (2004) 1304-1309.

[56] M. Velayutham, J.L. Zweier, Cardiac Ischemia and Reperfusion, in: Molecular Basis of Oxidative Stress, John Wiley & Sons, Inc., 2013, pp. 311-328.

[57] R. Franco, A. Garcia-Garcia, T.B. Kryston, A.G. Georgakilas, M.I. Panayiotidis, A. Pappa, Oxidative Stress and Redox Signaling in Carcinogenesis, in: Molecular Basis of Oxidative Stress, John Wiley & Sons, Inc., 2013, pp. 203-236.

[58] A. Federico, E. Cardaioli, P. Da Pozzo, P. Formichi, G.N. Gallus, E. Radi, Mitochondria, oxidative stress and neurodegeneration, J. Neurol. Sci, (2012).

[59] G. Durand, Synthetic Antioxidants, in: Molecular Basis of Oxidative Stress, John Wiley & Sons, Inc., 2013, pp. 377-406.

[60] C.E. Thomas, D.F. Ohlweiler, A.A. Carr, T.R. Nieduzak, D.A. Hay, G. Adams, R. Vaz, R.C. Bernotas, Characterization of the Radical Trapping Activity of a Novel Series of Cyclic Nitrone Spin Traps, J. Biol. Chem., 271 (1996) 3097-3104.

[61] C.E. Thomas, D.F. Ohlweiler, V.L. Taylor, C.J. Schmidt, Radical Trapping and Inhibition of Iron-Dependent CNS Damage by Cyclic Nitrone Spin Traps, J. Neurochem., 68 (1997) 1173-1182.

[62] D.A. Becker, J.J. Ley, L. Echegoyen, R. Alvarado, Stilbazulenyl Nitrone (STAZN): A Nitronyl-Substituted Hydrocarbon with the Potency of Classical Phenolic Chain-Breaking Antioxidants, J. Am. Chem. Soc, 124 (2002) 4678-4684.

[63] L. Yang, N.Y. Calingasan, J. Chen, J.J. Ley, D.A. Becker, M.F. Beal, A novel azulenyl nitrone antioxidant protects against MPTP and 3-nitropropionic acid neurotoxicities, Exp. Neurol., 191 (2005) 86-93.

[64] A. Dhainaut, A. Tizot, E. Raimbaud, B. Lockhart, P. Lestage, S. Goldstein, Synthesis, Structure, and Neuroprotective Properties of Novel Imidazolyl Nitrones, J. Med. Chem., 43 (2000) 2165-2175.

[65] B. Lockhart, A. Roger, N. Bonhomme, S. Goldstein, P. Lestage, In vivo neuroprotective effects of the novel imidazolyl nitrone free-radical scavenger (Z)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone (S34176), Eur. J. Pharmacol., 511 (2005) 127-136.

[66] M. Chioua, D. Sucunza, E. Soriano, D. Hadjipavlou-Litina, A. Alcázar, I. Ayuso, M.J. Oset-Gasque, M.P. González, L. Monjas, M.I. Rodríguez-Franco, J. Marco-Contelles, A. Samadi, α-Aryl-N-alkyl Nitrones, as Potential Agents for Stroke Treatment: Synthesis, Theoretical Calculations, Antioxidant, Anti-inflammatory, Neuroprotective, and Brain–Blood Barrier Permeability Properties, J. Med. Chem., 55 (2011) 153-168.

[67] A. Samadi, E. Soriano, J. Revuelta, C. Valderas, M. Chioua, I. Garrido, B. Bartolomé, I. Tomassolli, L. Ismaili, L. González-Lafuente, M. Villarroya, A.G. García, M.J. Oset-Gasque, J. Marco-Contelles, Synthesis, structure, theoretical and experimental in vitro antioxidant/pharmacological properties of α-aryl, N-alkyl nitrones, as potential agents for the treatment of cerebral ischemia, Bioorg. Med. Chem., 19 (2011) 951-960.

[68] Y. Sun, P. Yu, G. Zhang, L. Wang, H. Zhong, Z. Zhai, L. Wang, Y. Wang, Therapeutic effects of tetramethylpyrazine nitrone in rat ischemic stroke models, Journal of Neuroscience Research, 90 (2012) 1662-1669.

[69] F.A. Villamena, J.L. Zweier, Detection of reactive oxygen and nitrogen species by EPR spin trapping, Antiox. Red. Signal., 6 (2004) 619-629.

[70] M. Rosselin, F. Choteau, K. Zéamari, K.M. Nash, A. Das, R. Lauricella, E. Lojou, B. Tuccio, F.A. Villamena, G. Durand, Reactivities of Substituted α-Phenyl-N-tert-butyl Nitrones, J. Org. Chem., 79 (2014) 6615-6626.

[71] K. Reybier, T.H.Y. Nguyen, H. Ibrahim, P. Perio, A. Montrose, P.-L. Fabre, F. Nepveu, Electrochemical behavior of indolone-N-oxides: Relationship to structure and antiplasmodial activity, Bioelectrochemistry, 88 (2012) 57-64.

[72] T. Kubota, H. Miyazaki, Y. Mori, Bull. Chem. Soc. Jpn., 40 (1967) 245-251.

[73] C. Hansch, A. Leo, R.W. Taft, A survey of Hammett substituent constants and resonance and field parameters, Chem. Rev., 91 (1991) 165-195.

[74] P. Schmid, K.U. Ingold, Kinetic applications of electron paramagnetic resonance spectroscopy. 31. Rate constants for spin trapping. 1. Primary alkyl radicals, J. Am. Chem. Soc., 100 (1978) 2493-2500.

[75] C.L. Hawkins, M.J. Davies, Detection and characterisation of radicals in biological materials using EPR methodology, Biochimica et Biophysica Acta (BBA) - General Subjects, 1840 (2014) 708-721.

[76] C. Fréjaville, H. Karoui, B. Tuccio, F.L. Moigne, M. Culcasi, S. Pietri, R. Lauricella, P. Tordo, 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: A new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals, J. Med. Chem., 38 (1995) 258-265.

[77] G. Olive, A. Mercier, F. Le Moigne, A. Rockenbauer, P. Tordo, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide: evaluation of the spin trapping properties, Free Radical Biol. Med., 28 (2000) 403-408.

[78] H. Zhao, J. Joseph, H. Zhang, H. Karoui, B. Kalyanaraman, Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals, Free Radical Biol. Med., 31 (2001) 599-606.

[79] A. Allouch, V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, Spin trapping of superoxide by diester-nitrones, Org. Biomol. Chem., 3 (2005) 2458-2462.

[80] V. Roubaud, R. Lauricella, J.-C. Bouteiller, B. Tuccio, N-2-(2-Ethoxycarbonyl-propyl) [alpha]-Phenylnitrone: An Efficacious Lipophilic Spin Trap for Superoxide Detection, Archives of Biochemistry and Biophysics, 397 (2002) 51-56.

[81] T.L. Fevig, S.M. Bowen, D.A. Janowick, B.K. Jones, H.R. Munson, D.F. Ohlweiler, C.E. Thomas, Design, Synthesis, and in Vitro Evaluation of Cyclic Nitrones as Free Radical Traps for the Treatment of Stroke, Journal of Medicinal Chemistry, 39 (1996) 4988-4996.

[82] C.P. Sár, É. Hideg, I. Vass, K. Hideg, Synthesis of α-aryl N-adamant-1-yl nitrones and using them for spin trapping of hydroxyl radicals, Bioorganic & Medicinal Chemistry Letters, 8 (1998) 379-384.

[83] E. Hideg, A. Takátsy, C.P. Sár, I. Vass, K. Hideg, Utilizing new adamantyl spin traps in studying UV-B-induced oxidative damage of photosystem II, Journal of Photochemistry and Photobiology B: Biology, 48 (1999) 174-179.

[84] W. Porcal, P. Hernández, M. González, A. Ferreira, C. Olea-Azar, H. Cerecetto, A. Castro, Heteroarylnitrones as Drugs for Neurodegenerative Diseases: Synthesis, Neuroprotective Properties, and Free Radical Scavenger Properties, Journal of Medicinal Chemistry, 51 (2008) 6150-6159.

[85] R. Soto-Otero, E. Méndez-Álvarez, S. Sánchez-Iglesias, J.L. Labandeira-García, J. Rodríguez-Pallares, F.I. Zubkov, V.P. Zaytsev, L.G. Voskressensky, A.V. Varlamov, M. de Candia, F. Fiorella, C. Altomare, 2-Benzazepine Nitrones Protect Dopaminergic Neurons against 6-Hydroxydopamine-Induced Oxidative Toxicity, Archiv der Pharmazie, 345 (2012) 598-609.

[86] Y. Han, B. Tuccio, R. Lauricella, A. Rockenbauer, J.L. Zweier, F.A. Villamena, Synthesis and Spin-Trapping Properties of a New Spirolactonyl Nitrone, J. Org. Chem., 73 (2008) 2533-2541.

[87] T. Kolter, G. van Echten-Deckert, K. Sandhoff, Synthesis of sphinganine analogues modified in the head group, Tetrahedron, 50 (1994) 13425-13432.

Chapter III

Towards the improvement of the Bioavailability of Nitrones

Chapter III – Part 1

Multivalence and Synergistic effects of antioxidants: Development of a new fluorinated amphiphilic carrier

I. Introduction

Bioavailability of antioxidants is of particular interest in the development of new compounds with improved protective activity against oxidative stress-induced damage. For several years, our laboratory has been interested in improving the protective activity of nitrone-type synthetic antioxidants. Our strategy is based on the modulation of the hydrophilic/lipophilic balance of the nitrone-containing derivatives, in order to ensure an enhanced membrane crossing ability. The amphiphilic character of these nitrones has therefore been demonstrated to improve the bioavailability and the protection against oxidative stress.[1-4]

The design of hybrid molecules including two pharmacophores in one molecular scaffold is currently being investigated for several types of drug and active compounds. The hybrid approach consists in the conjugation of two or more different active moieties in order to provide improved properties to the molecule.[5],[6] This has been used in the field of antioxidants molecules. For example, the synergistic interactions between lipoic acid and other antioxidants were described by several groups and were generally found to be beneficial for the antioxidant activity.[7-9] Hybrids compounds combining the α-tocopherol chroman moiety and other antioxidants were also synthesized, demonstrating that the antioxidant activity greatly depend upon the synergistic action of the two different antioxidants.[10-12] More recently, Hadjipavlou-Litina and co-workers have designed compounds with high inhibition of soybean lipoxygenase (LOX) by conjugation of different antioxidant scaffolds.[13]

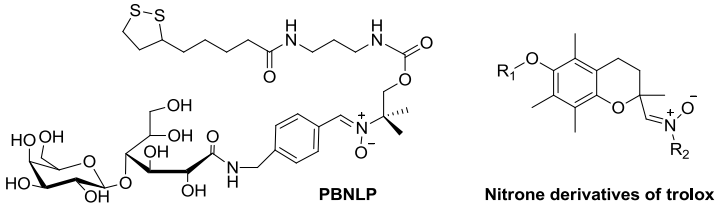


Figure 3.1.1. Exemples of hybrid antioxidants.

Therefore, the synthesis of hybrid molecules bearing one nitrone moiety and another type of antioxidant appears to be a promising strategy as it allows to combine in one unified molecular scaffold, two antioxidant moieties with particular properties. Our group previously developed an amphiphilic hybrid of lipoic acid and PBN, called PBNLP (Figure 3.1.1), in which the nitronyl group is the linker between the lipophilic lipoic acid and the polar head group.[14] *In vitro* protection afforded by PBNLP against AAPH-induced hemolysis of erythrocytes demonstrated the beneficial effect of chemical association of a water soluble derivative of PBN and lipoic acid compared to these two derivatives in admixture and, to PBN and lipoic acid alone. It was suggested that the amphiphilic character of PBNLP partially governs the beneficial effect by increasing the bioavailability and by reducing the negative chemical interactions between the two compounds. Balogh and co-workers have also synthesized trolox-nitrone conjugates (Figure 3.1.1) whose antioxidants properties have been evaluated *in vitro* and *in vivo*.[15] The conjugates exhibited a comparable activity as Trolox against *in vitro* lipid peroxidation and exceeded nitrone-type references PBN and NXY-059 in a mice model of permanent focal ischemia.

As an ongoing effort towards the design of highly potent antioxidants, we have decided to synthesize new hybrid amphiphilic antioxidants using the optimized amphiphilic carrier developed in our laboratory and composed of a sugar-based polar head, a perfluorinated hydrophobic tail and a lysine amino-acid as central scaffold upon which the antioxidant moiety can be grafted through an amide bond.[4] By combining two lysine amino acids, the synthesis of multivalent antioxidant derivative was made possible. We first worked on the synthesis of a divalent amphiphilic derivative in which two PBN moieties were grafted onto the lysine side chains of the carrier. This compound is called FADiPBN, FA meaning "Fluorinated Amphiphilic carrier" and Di denoting the presence of two nitrone moieties. By comparing this derivative to its monovalent derivative FAPBN,[3, 4] we can study the effect

of the multi-valence effect on the antioxidant properties. We then extended our work on the search for a synergistic effect between different antioxidants grafted onto the same carrier. This led us to develop a hybrid derivative bearing both PBN and Trolox moieties. This derivative was called using our nomenclature FATxPBN, Tx denoting the Trolox group.

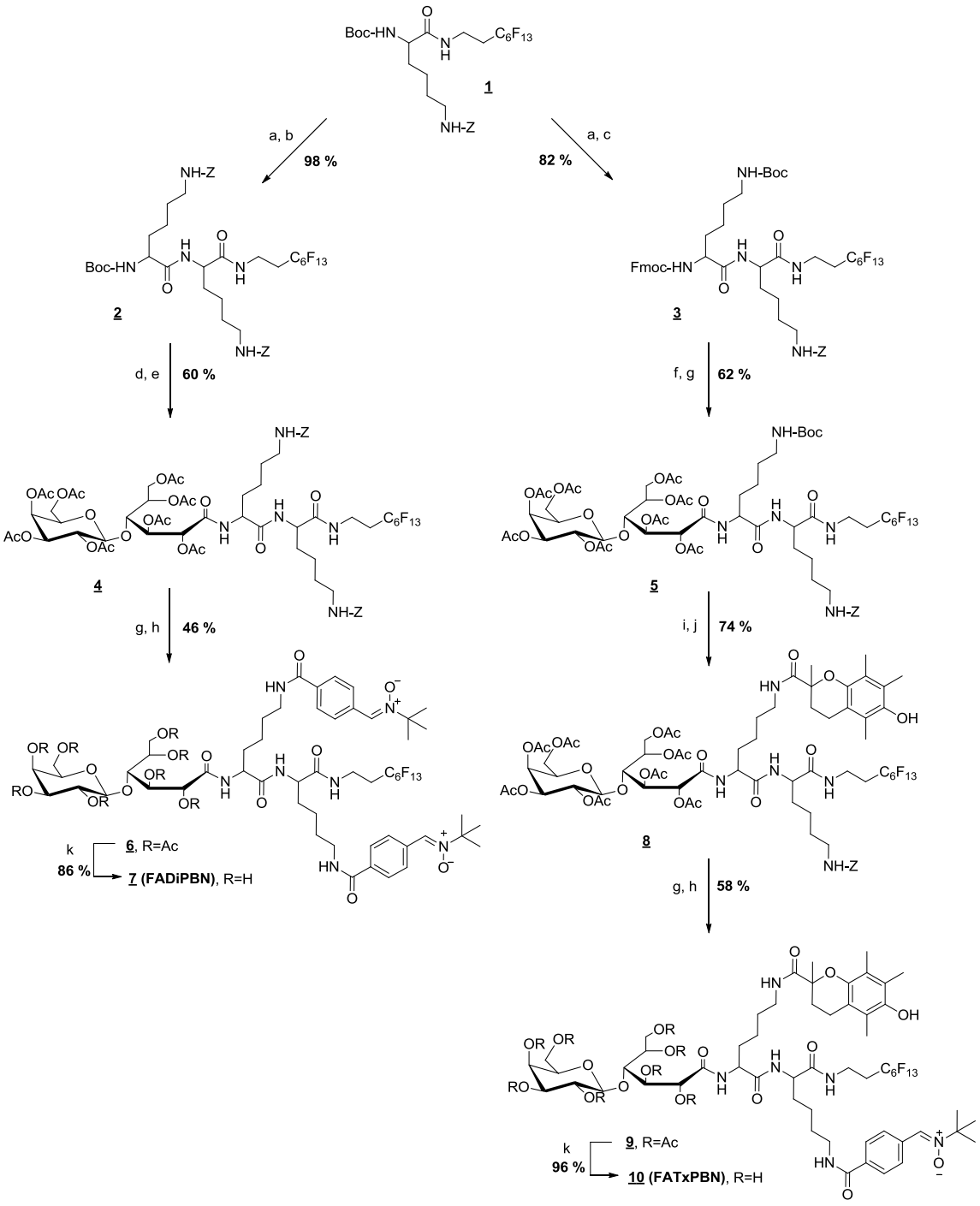
In the search for new amphiphilic derivatives, we also developed compounds in which the sugar-based polar head was replaced either by an anionic (carboxylate) or a cationic (ammonium) polar head group so as to obtain pH-dependent derivatives. With the anionic carboxylate derivative, one can expect to obtain a better solubility at high pH whereas with the cationic ammonium compound one should obtain better solubility at low pH. In biology, pH is of particular importance, the physiological pH in living system is around 7.4 but differs in the several cellular compartment or organs. Therefore, having these two ionic compounds in hand would allow us to work on a large range of pH. For the sake of comparison, their corresponding protected derivatives bearing one or two PBN moieties were also synthesized in order to study their physical-chemical properties as well as antioxidant and anti-inflammatory activities.

II. Results and discussion

1. Synthesis of the divalent antioxidants FADiPBN and FATxPBN.

Synthesis of the DiLysine carriers 4 and 5. The synthesis starts from compound **1,** which was prepared following our established procedure[4] from the commercially available 1H,1H,2H,2H-perfluoroctyl iodide. First, deprotection of compound **1** was carried out in a dichloromethane solution containing 20% of trifluoroacetic acid (TFA) and the resulting amino form was directly used without purification. On the one hand, Boc-Lys(Z)-OH was grafted on the deprotected compound **1** using the classical *N*,*N*'-Dicyclohexylcarbodiimide (DCC) reagent in presence of a catalytic amount of hydroxybenzotriazole (HOBt), in dry dichloromethane to obtain compound **2** in 98% yield. In parallel, the lactobionolactone polar

head was prepared by deshydratation of lactobionic acid in acidic media following a procedure routinely used in our laboratory. Compound **2** was then deprotected in acidic conditions and lactobionolactone was added to the resulting amino compounds in solution in Ethanol with a few drops of TEA (pH ~ 8-9). The mixture was stirred at 60-70°C until complete consumption of the amino compound and was then acetylated using Ac_2O/Pyridine (1/1 v:v). After purification by flash chromatography, compound **4** was obtained in 60% (Scheme 3.1.1). On the other hand, the second carrier was easily obtained following a connected procedure. The only difference lies in the second lysine moiety which was protected with a fluorenylmethyloxy carbonyl group (Fmoc) on the C-terminal part and with a Boc group on the side chain. This led to compound **3** in 82% yield. The corresponding amphiphilic carrier (**5**) was then obtained in 62% yield (Scheme 3.1.1).



Scheme 3.1.1. Synthesis of FADiPBN and FATxPBN. a) CH_2Cl_2/TFA (8:2); b) Boc-Lys(Z)-OH, DCC, HOBt, CH_2Cl_2; c) Fmoc-Lys(Boc)-OH, DCC, HOBt, CH_2Cl_2; d) Lactobionolactone, EtOH, 60°C; e) Ac_2O/Pyridine; f) CH_3CN/DEA (9:1); g) H_2, Pd/C, EtOH/AcOH (99:1), 5 bars; h) HOSu-PBN, dry CH_2Cl_2; i) Trolox®, DCC, HOBt, dry CH_2Cl_2; j) MeONa, MeOH.

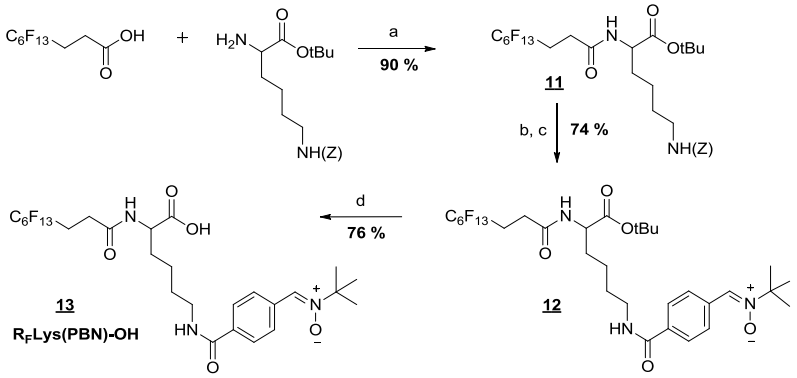
Synthesis of FADiPBN (7). The two protecting groups of compound **4** were removed by hydrogenolysis using a catalytic amount of 10% Pd/C in ethanol/acetic acid (99:1 v/v) and submitted to a hydrogen atmosphere of 5 bars. After filtration of the catalyst through a pad of Celite, the resulting amino compound was added to a solution of HOSu-PBN[4] in dry dichloromethane at room temperature and under argon atmosphere. After purification by flash chromatography followed by size exclusion chromatography, the acetylated compound **6** was obtained in 46% yield in the two steps. Finally removal of the acetyl groups by Zemplén desacetylation in presence of a catalytic amount of sodium methoxide in dry methanol led, after purification on size exclusion chromatography, to FADiPBN in 86% yield. The overall yield for the preparation of FAPBN is 16 % in 5 steps.

Synthesis of FATxPBN (10). The Boc protecting group of compound **5** was first removed using 20% of TFA in dry CH_2Cl_2 and the resulting amino compound was directly coupled to Trolox® in the presence of DCC and HOBt in dry CH_2Cl_2. Previous work in our group showed that the preparation of activated ester of Trolox was troublesome.[4] That is why we rather used the classical DCC/HOBt coupling system. After purification by flash chromatography, compound **8** was obtained in 74% yield. Then, the carboxyphenyl group (Z) was deprotected by hydrogenolysis following the same procedure as described above and the resulting amino compound was added to a solution of HOSu-PBN[4] in dry dichloromethane at room temperature and under argon atmosphere. After purification by flash chromatography followed by size exclusion chromatography, the acetylated compound **9** was obtained in 58% yield. Finally, deprotection of the hydroxyl groups following Zemplén transeterification led after purification by size exclusion chromatography to FATxPBN in 96% yield. The overall yield for the preparation of FATxPBN was 15 % in 6. Both FADiPBN and FATxPBN were characterized by ^{1}H, ^{13}C and HSQC NMR experiments as well as mass spectroscopy. The purity (98%>) was then confirmed by C18 reverse phase HPLC at 290 nm.

2. Synthesis of the ionic antioxidant derivatives. The main characteristic of a carboxylate- or an amino-based amphiphile is that depending on the pH of the solution, it can be either charged or uncharged altering thereby its solubility in water. Two ionic antioxidant derivatives with a carboxylate and an amino group were synthesized (Schemes 3.1.2 and 3.1.3) and were respectively called and R_F-Lys(PBN)-OH and H-Lys(PBN)-R_F, "R_F" denoting the perfluorinated chain -$CH_2CH_2C_6F_{13}$.

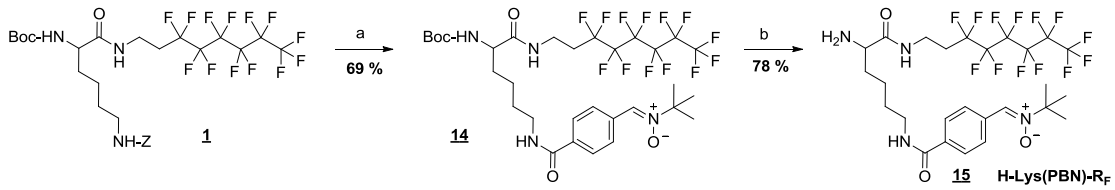
Synthesis of R_F-Lys(PBN)-OH (13). The anionic derivative was easily obtained in four steps. Firstly, the commercially available 2*H*,2*H*,3*H*,3*H*-Perfluorononanoic acid was grafted on the free amino group of H-Lys(Z)-OtBu in dry dichloromethane using DCC/HOBt. After purification, compound **11** was obtained in 90% and was then submitted to a 5 bars hydrogen atmosphere with a catalytic amount of 10% Pd/C. The activated ester HOSu-PBN[4] was grafted onto the resulting amino derivative and after purification, compound **12** was obtained in 74% in the two steps. Finally, removal of the *tert*-butyl ester protecting group was carried out in acidic condition and appeared the most sensitive step. Indeed, the nitronyl group is rather sensitive and can easily decompose in aqueous solution under acidic conditions to give the corresponding benzaldehyde and *N*-*tert*-butyl hydroxylamine, as previously reported in the literature.[16, 17] To avoid degradation of the nitrone function, we first tried to remove the ester group with 20% of TFA in dry dichloromethane. TLC monitoring and ^{1}H NMR spectroscopy showed the complete absence of deprotection, even after 3 h of stirring. The concentration of TFA was then increased to 30% and reaction time to 6 h but the deprotection was only partial. Complete deprotection of the amino group was obtained with 50% of TFA in dry dichloromethane. However, ^{1}H NMR spectroscopy showed the presence of around 20-30 % of benzaldehyde by-product, arising from the cleavage of the nitronyl function, which was difficult to purify because of the relatively close retention factor to that of the nitrone derivative. To avoid the cleavage of the nitronyl group, tetraethylsilane (TES) was used as a

scavenger of the carbocation formed during the reaction. Finally, after a rapid screening of the experimental condition, the best one was the use of a solution of TFA/CH_2Cl_2 (5:5, v/v) with 2 equivalents of TES at 0°C and under argon atmosphere. After 3 h of stirring, the crude mixture was purified by flash chromatography eluted with EtOAc/MeOH (8:2, v/v) containing 1% of AcOH and R_F-Lys(PBN)-OH was obtained in 76% yield.



Scheme 3.1.2. Synthetic pathway of Rf-Lys(PBN)-OH. a) DCC/HOBt/CH_2Cl_2; b) H_2, Pd/C, EtOH; c) HOSu-PBN, CH_2Cl_2; d) CH_2Cl_2, TFA (5:5, v/v), TES.

Synthesis of H-Lys(PBN)-R_F (15). The synthesis starts from compound **1** whose side chain was deprotected under hydrogen atmosphere with a catalytic amount of 10% Pd/C. An excess of active ester HOSu-PBN[4] was then added to the resulting amino compound in solution with dry CH_2Cl_2 and after purification, compound **14** was obtained in 69% yield. Like for the synthesis of the anionic compound, the deprotection of the amino group was difficult with the formation of by-products even in the presence of TES scavenger. After several assays, the best condition was found to be CH_2Cl_2/TFA (8:2, v/v) at 0°C with 2 equivalents of TES. Purification by flash chromatography eluted with EtOAc/MeOH (9:1, v/v), led to H-Lys(PBN)-R_F in 78% yield.



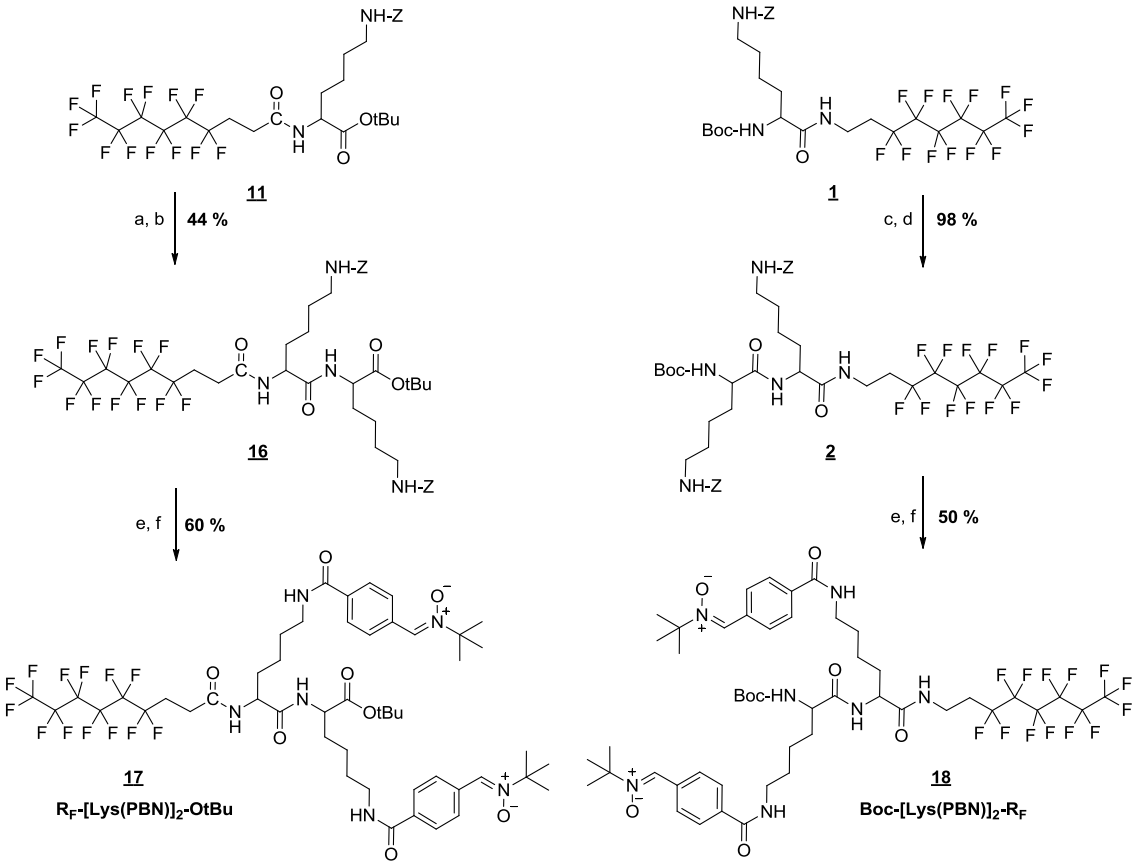
Scheme 3.1.3. Synthetic pathway of H-Lys(PBN)-R_F. a) H_2, Pd/C, EtOH; b) HOSu-PBN, CH_2Cl_2; d) CH_2Cl_2, TFA (8:2, v/v), TES.

3. Synthesis of uncharged lipophilic antioxidant derivatives. We were also interested in the synthesis of divalent ionic derivatives with either a carboxylate or an amino group. We therefore developed another synthetic strategy as described in Scheme 3.1.4. However, the last step consisting in the removal of the protective groups failed to provide the desired compounds in spite of several attempts. We report herein the synthesis until to the penultimate step. This led us to two lipophilic divalent derivatives bearing two PBN moieties, denoted as R_F-[Lys(PBN)]$_2$-OtBu (**17**) and Boc-[Lys(PBN)]$_2$-R_F (**18**) that can be compared to the univalent derivatives, respectively compounds **12** and **14**.

Synthesis of R_F-[Lys(PBN)]$_2$-OtBu (17). Starting with compound **11**, the first step consisted in the removal of the ester protecting group in acidic conditions. The carboxylic acid group of the resulting compound was then coupled with H-Lys(Z)-OtBu in presence of DCC and a catalytic amount of HOBt in dry dichloromethane with few drops of TEA (pH~8-9) to obtain, after purification, compound **16** in 44% yield. With regard to the relatively low yield, a second assay was carried out with 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (TBTU) as a coupling reagent in the presence of DIEA in dry dichloromethane but no significant improvement of the yield was observed. Then the two carboxybenzyl groups were simultaneously removed under hydrogen atmosphere with a catalylic amount of 10% Pd/C. The active ester of PBN was grafted on the two amino functions of the resulting compound to give R_F-[Lys(PBN)]$_2$-OtBu in 60% yield.

Some assays were done to remove the *tert*-butyl protecting group and obtain an amphiphilic pH-dependant compound bearing two PBN moieties but results were unsuccessful. Because of the two nitrone functions present, a wide range of by-products were obtained and after purification, no fraction of the desired product was observed. We decided to study the properties of the protected compound itself. We will further pursue our efforts to synthesize the corresponding deprotected compound.

Synthesis of Boc-[Lys(PBN)]$_2$-R$_F$(18). Compound **2** was synthesized from compound **1**. The two lysine side chains were deprotected using a catalytic amount of 10% Pd/C in EtOH solution and under hydrogen atmosphere. The active ester of PBN was then grafted on the two resulting amino functions to give after purification by flash chromatography and by size exclusion chromatography, compound Boc-[Lys(PBN)]$_2$-R$_F$ in 50% yield. In light of the degradations observed when removing the protecting group in acidic conditions, we choose not to deprotect it and study the physical chemical and biological properties of the protected form.



Scheme 3.1.4. Synthetic pathway of R$_F$-[Lys(PBN)]$_2$-OtBu and Boc-[Lys(PBN)]$_2$-R$_F$. a) CH_2Cl_2, TFA (5:5, v/v); b) H-Lys(Z)-OtBu/DCC/HOBt/CH_2Cl_2; c) CH_2Cl_2, TFA (8:2, v/v); d) Boc-Lys(Z)-OH/DCC/HOBt/$CH_2Cl_2H_2$, e) H_2, Pd/C, EtOH; f) HOSu-PBN, CH_2Cl_2.

Physical-chemical measurements.

The three non-ionic amphiphilic FAPBN, FADiPBN and FATxPBN were freely soluble in water (~10 $g.L^{-1}$) whereas R_F-Lys(PBN)-OH and H-Lys(PBN)-R_F were insoluble in water and were therefore studied at different pH solutions. The theoretical p*K*a of the acidic function of lysine is 2.18 suggesting that at higher pH value, the carboxylate form should predominate, therefore favoring the solubility of the amphiphilic compound. However, experiments showed that a 1mM solution of R_F-Lys(PBN)-OH was, neither in pH 4 buffer nor in ultrapure water, soluble. Few drops of a 15 mM sodium hydroxide solution (NaOH) were slowly added to that solution in order to favor deprotonation of the carboxylic acid and afford complete solubilization. R_F-Lys(PBN)-OH was found freely soluble at pH~9-10 and after hand shaking, the characteristically foam of a solution of an amphiphilic compound was observed. To study the stability of the sensitive PBN moiety, a solution of PBN at pH ~10 was followed by NMR spectroscopy. After one week in solution, no degradation of the nitrone was observed. Concerning the amine function of lysine, the theoretical p*K*a is 8.95 suggesting that below this value, the ammonium form should predominate, favoring the solubility of the amphiphilic compounds. H-Lys(PBN)-R_F was, neither in pH 4 buffer nor in ultrapure water, soluble. A more acidic buffer was prepared using KCl and HCl in water solution and the cationic compound **15** was found soluble at pH ~2. Because of the nitrone sensitivity to acidic solution, solution of compound **15** in that pH ~2 buffer was analyzed by ^{1}H NMR spectroscopy and hydrolysis of the nitrone function into its corresponding aldehyde and hydroxylamine derivatives was observed. The insolubility of H-Lys(PBN)-R_F above pH ~4 and the degradation observed at pH ~2 greatly limits its use as a water soluble compound.

Determination of partition coefficients (log k'_w). All the newly synthesized compounds were found to be more lipophilic than the parent PBN due to the presence of the hydrophobic fluorinated chain. When comparing the mono and divalent derivatives, we showed that the

lipophilcity is slightly increased for the di-lysines derivatives. Indeed, a partition coefficient of 4.92 was found for FADiPBN while that of FAPBN is 4.30. This was also confirmed for compounds **17** and **18** compared to compounds **12** and **14**, respectively. The high lipophilicity of FATxPBN arises from the presence of the lipophilic Trolox moiety, leading to a log k'_w value of 6.37. We also observed that position of the fluorinated group on the amino acid *i.e.* amino or carboxylic acid group has no significant effect on the lipophilicity. Lipophilicity of the anionic compound **13** was found lower than its corresponding protected compound **12**, due to the free carboxylic acid function which confers hydrophilicity. The partition coefficient of H-Lys(PBN)-R_F was not determined due to its insolublility in methanol. Results are summarized in Figure 3.1.2.

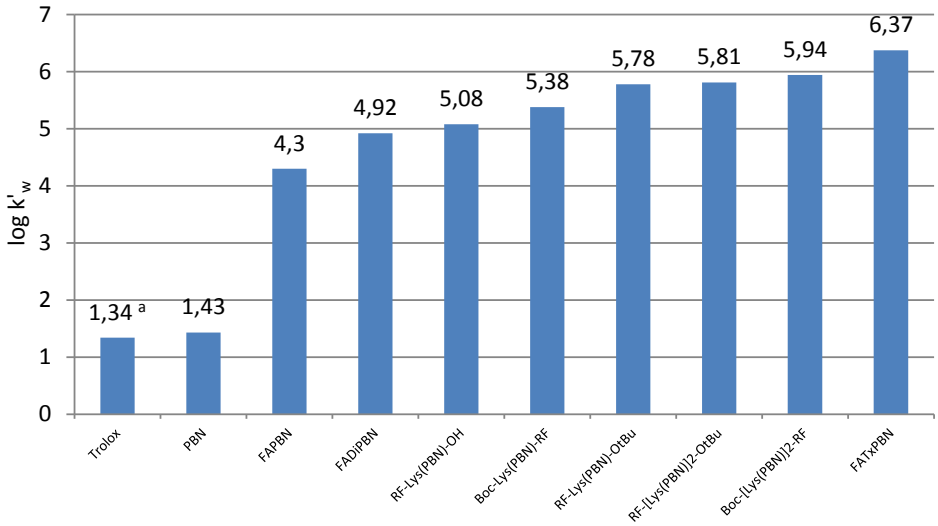


Figure 3.1.2. Lipophilicity values of nitrone derivatives. [a] Values from the literature.[4]

Critical micelle concentration (CMC). ^{19}F NMR spectroscopy was used to determine the concentration above which micelles are formed. Among the experimental techniques available for the study of micelle formation of fluorinated surfactants, the ^{19}F NMR offers the advantage of being rapid without requiring large amount of samples. It has been used to study the mixed surfactant systems as it allows to give information on the composition of the mixed micelles.[18] The chemical shift of the CF_3 group varies under isotropic or anisotropic

conditions, therefore when plotting the chemical shifts of the group with the concentration; one can observe a transition indicating the CMC is reached. A concentrated solution of FAPBN, FADiPBN and FATxPBN (~1 to 3 mM) was prepared in H_2O/D_2O (9:1, v/v) and dilutions were then made with this H_2O/D_2O, using a trifluoroacetate salt as a reference. For FAPBN, the intersection of the two linear curves gave a CMC of ~ 0.33 mM, whereas a lower value of ~ 0.05 mM was obtained by the surface tension method.[4] For FADiPBN and FATxPBN, no well-defined transition was observed between the anisotropic and isotropic regimes as it was observed for FAPBN. We plotted the chemical shift versus log *C* as it is commonly done for surface tension measurements. As shown in Figure 3-3B, two domains of concentration where the chemical shit was constant were observed with in between a drop of the shift of ~ 2 ppm. The CMC was therefore approximated by averaging the two closest concentration of the chemical shift change; leading to value of ~ 0.30 mM for FADiPBN and ~ 0.40 mM for FATxPBN. This indicates that FAPBN and FADiPBN both self-assemble at concentration near to 0.3 mM suggesting that the incorporation of a second linker bearing PBN have no significant effect on self-agregation properties, which is an agreement with the close log k'_W values observed. However for FATxPBN the value of 0.40 mM looks suspicious. We previously reported a CMC value of 0.025 mM determined by surface tension for the FATx derivative, the univalent lysine derivative with one Trolox moiety,[4] which indicates higher hydrophobicity of the Trolox derivatives compared to the PBN ones. The opposite trend observed here when comparing FATxPBN and FADiPBN is therefore rather surprising and indicated that further experiments must be conducted as this result arises from only one experiment.

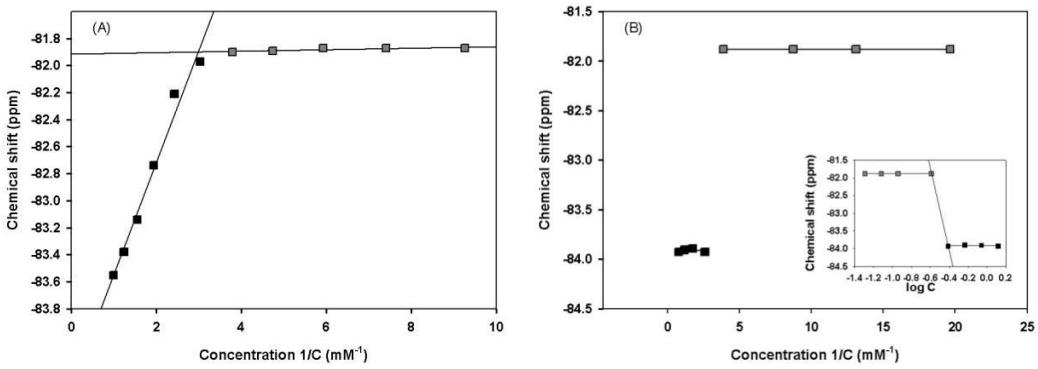


Figure 3.1.3. (A) CMC measurement of FAPBN; (B) CMC measurement of FADiPBN, inset is shown corresponding logarithmic values.

Determination of the CMC of R_F-Lys(PBN)-OH was carried out using at pH=10 in order to have the compound freely soluble. The higher lipophilicity of R_F-Lys(PBN)-OH compared to FAPBN as demonstrated by the higher log k'_W value of the former compound suggests it should exhibit a lower CMC value as well. Surprisingly, we found that compound **13** self-aggregates at a concentration of ~1.1 mM which is 3-fold that of FAPBN (The experiment was done in triplicate). One hypothesis is that the high pH of the solution (10) necessary to ensure a complete solubility of compound **13** may alter the CMC value compared to that of FAPBN measured in pure water. This point will be investigated in the near future. Finally, the CMC of H-Lys(PBN)-R_F was not investigated because of its insolubility and instability.

Dynamic light scattering (DLS). The self-assemblies properties of FAPBN, FADiPBN and R_F-Lys(PBN)-OH were determined in water by DLS at 25°C. All compounds were studied at a concentration higher than 10 × CMC. We showed that both FAPBN and FADiPBN self-assemble into compact and well-defined micelles of ~8 nm diameter, demonstrating that the presence of a second lysine scaffold has no influence on the self-assembly properties (Figure 3.1.4.A). This is in agreement with our previous observation where we measured an hydrodynamic diameter of ~6 nm for FAPBN.[3] We also investigated the effect of the pH on the self-aggregation of R_F-Lys(PBN)-OH with the non-ionic FAPBN as a reference. We observed that at pH=4, 6, 8 and 10, FAPBN self-assembles into stable and compact micelles

with a slight increase of the particle size when increasing the pH. Repeated Contin analysis of FAPBN showed a hydrodynamic diameter of ~12 nm at pH=10, with a negligible population of bigger particles (Figure 3.1.4.B, inset). It should be noted that, although a bimodal pattern was observed in the intensity-weighed particle size distribution, the larger particles account for only a small fraction of the total material present in the samples; otherwise the scattering contributions from these particles would have completely masked those from the smaller particles. In the relevant size range, the scattering intensity scales with d^6, a 200 nm particle thus scatters ~4×10^9 times more light than a 5 nm particle. On the contrary, the anionic compound R_F-Lys(PBN)-OH showed larger aggregates of ~200 nm in diameter. As illustrated with the ill-defined correlation function (Figure 3.1.4.C) the aggregates formed are heterogenous and rather unstable.

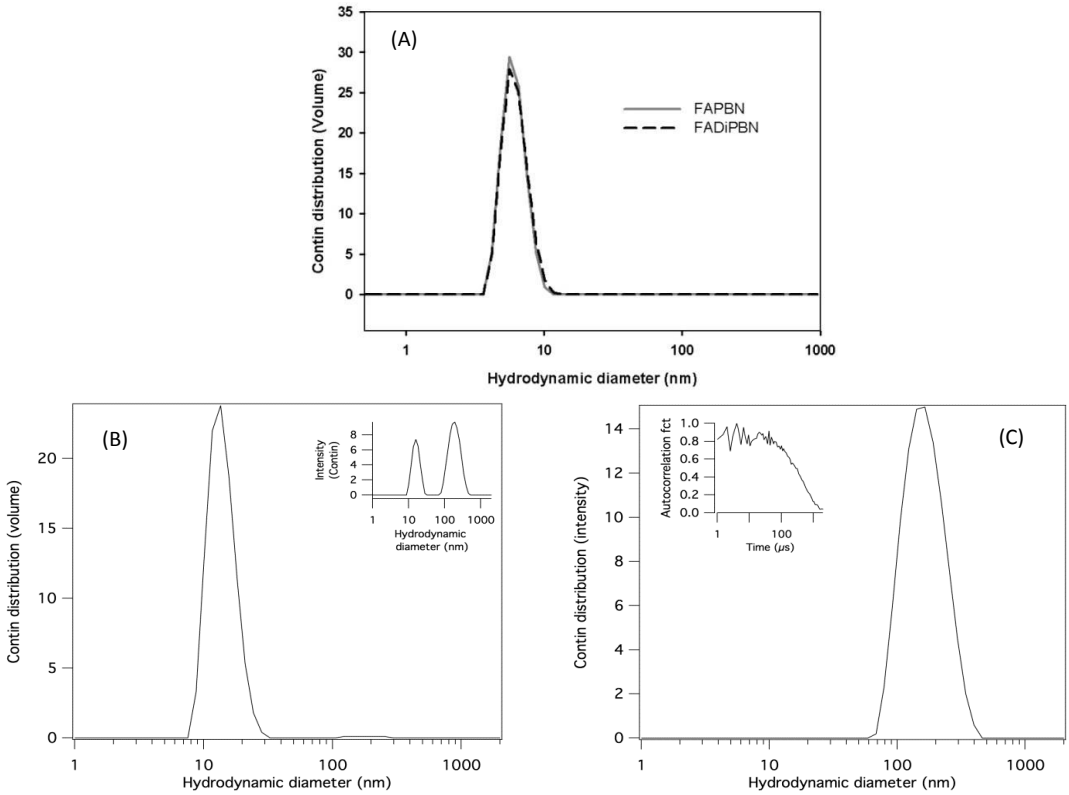


Figure 3.1.4. Contin analysis (volume percentage) of (A) FAPBN and FADiPBN in water and (B) FAPBN in water (pH=10); inset is shown intensity contin distribution. Intensity Contin distribution of R_F-Lys(PBN)-OH in water (pH=10); inset is shown autocorrelation function (C).

Electronic Paramagnetic Resonance (EPR) spectroscopy. The spin-trapping properties of amphiphilic nitrones derivatives were studied using Electron Paramagnetic Resonance (EPR) spectroscopy. The two amphiphilic derivatives bearing one or two PBN moiety were used to study the influence of the amphiphilic carrier as well as the presence of the second nitrone function on the spin-trapping properties. The methoxy radical was chosen because of its easiness to be generated using methanol, dimethylsufoxide and lead tetraacetate. A solution of nitrone (20 mM) in DMSO containing 10% v/v of MeOH was prepared and ~1 mg of solid $Pb(OAc)_4$ was added. In these conditions, the methoxy spin-adduct of FAPBN and FADiPBN was immediately observed by EPR spectroscopy (Figure 3.1.5).

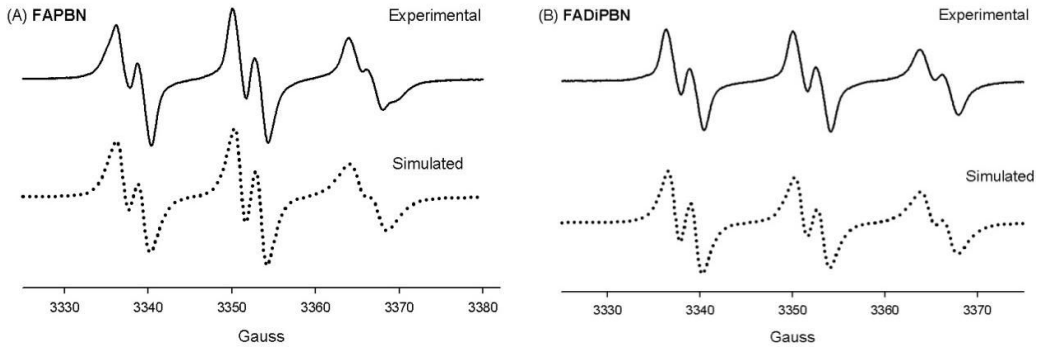


Figure 3.1.5. EPR spectrums of FAPBN (A) and FADiPBN (B) methoxy spin-adducts.

For both derivatives, a six lines spectrum was obtained due to the hyperfin coupling of the electron with the nitrogen giving three lines which are split because of hyperfine interactions with the hydrogen in β position of the nitrone function. Simulation of the experimental results led to hyperfine splitting coupling constants of a_N = 14.04 G and a_H = 2.44 G for FAPBN while for FADiPBN we obtained a_N = 13.74 G and a_H = 2.30. These results are in good agreement with the literature for methoxy spin-adduct of linear nitrones. This shows that despite the steric hindrance brought by the amphiphilic carrier, the nitronyl function keeps its ability to trap free radicals. However, experimental spectra showed broad and asymmetric line width which is characteristic of a slow molecular tumbling motion witnessing anisotropic

conditions. This restricted rotational motion of the spin-adducts has already been observed for amphiphilic nitrones and was explained as a result of the micellar aggregate formation.[2, 3]

Cyclic voltammetry (CV). The electrochemical characterization of FAPBN and FADiPBN was investigated using cyclic voltammetry (CV) both in aqueous and organic solutions. We first carried out cyclic voltammetry in aqueous conditions with 50 mM of NaCl as electrolyte. An irreversible one-step reduction of about -1.74 V vs. Ag/AgCl was observed for PBN in aqueous conditions, in agreement with values obtained by McIntire et al.[19] A higher cathodic potential was obtained for FAPBN and FADiPBN with values of -1.46 V and -1.48 V, respectively (Figure 3.1.6). This indicates that once grafted onto the carrier the nitronyl group is more easily reduced. On the contrary, no oxidation potentials were detected in aqueous solution, as previously observed for other nitrones.[19, 20]

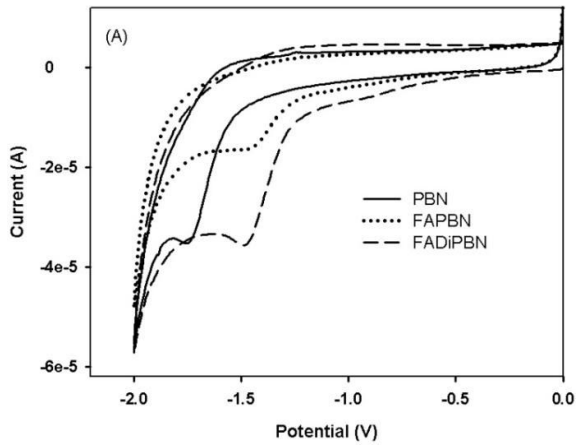


Figure 3.1.6. Reduction of PBN, FAPBN and FADiPBN in 50 mM NaCl at 0.1 V/s.

The electrochemical properties of these three compounds were next study in non-aqueous media using tertabutylamonium perchlorate (TBAP) as electrolyte. As previously observed for the *para*-substituted nitrone series (see chapter 2), two reduction potentials were observed for PBN whose difference from each other was 0.18 ± 0.02 V. For FAPBN and FADiPBN, two peaks were also observed however with higher difference. Reduction potentials of the nitronyl group were assigned to the peak at -1.87 V for FAPBN and at -1.89 V for FADiPBN

whereas the second peak, observed at lowest potentials, may witness the contribution of the amphiphilic carrier (Figure 3.1.7.A). Oxidative potentials were then determined in acetonitrile and no potentials were observed for FAPBN and FADiPBN at scan field between 0 and 2.0 V. When increasing the potential window until 3.0 V, two potential values of 1.90 V and 2.58 V were obtained for FAPBN. According to the data observed for the PBN derivatives presented in chapter 2, the oxidation potential of 1.90 V was assigned to the nitronyl function whereas the highest peak was assigned to the amphiphilic carrier. For FADiPBN, however, only one peak was observed at 2.56 V, which might indicate that the oxidation potential of the nitronyl function is hidden under the highest peak potential (Figure 3.1.7.B).

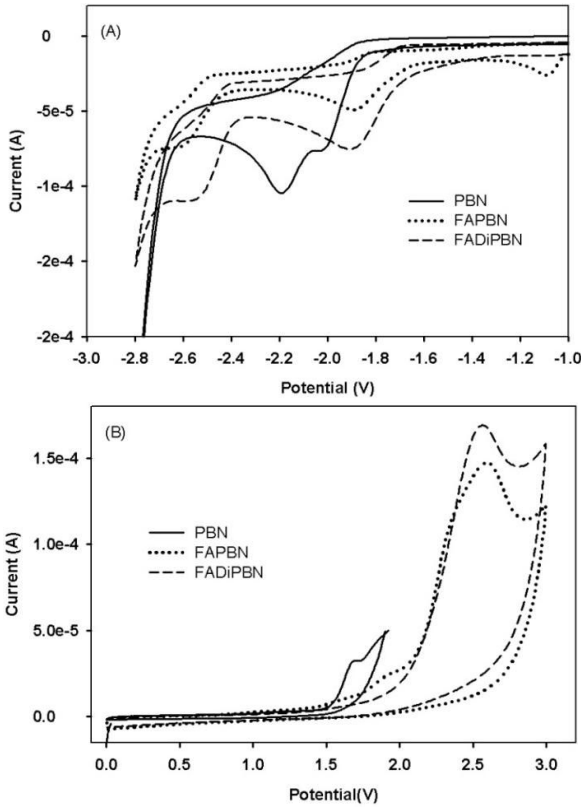


Figure 3.1.7. Reduction (A) and Oxidation (B) of PBN, FAPBN and FADiPBN in acetonitrile containing 50mM of TBAP at 0.1 V/s.

In the second chapter of this manuscript, we have demonstrated that *para*-substitution on the phenyl group of PBN moiety with electron-donating or electron-withdrawing substituents have an influence on the electrochemical properties of the nitronyl group. Compounds bearing

an electron-withdrawing group in *para*-position are more easily reduced and hardly oxidized than those having an electron-donating substituent, the effect being linearly correlated with the Hammett sigma para constants (σ_p). For both FAPBN and FADiPBN, the PBN moiety is grafted onto the carrier through an amide bond which is slightly electro-withdrawing. The oxidation and reduction potentials observed here for FAPBN and FADiPBN support our observations that electron-withdrawing substitutions in *para*-position of the phenyl ring lead to more easily reduced and hardly oxidized compounds both in aqueous or non-aqueous media.

In vitro antioxidant activity and radical scavenging activity study. In collaboration with the School of Pharmacy, Aristotle University of Thessaloniki, the *in vitro* antioxidant activity of FAPBN, FADiPBN, Boc-Lys(PBN)-R_F, Boc-Lys(PBN)-Lys(PBN)-R_F and R_F-Lys(PBN)-OH was evaluated. Several assays are used in order to assess *in vitro* antioxidant activity because the antioxidant ability of a compound must be evaluated in a variety of milieus. Factors such as solubility or steric hindrance may be of overriding importance in different environments. Each method relates to the generation of a different radical.[21] In this work, two types of assays were employed. The first assay is based on the scavenging reduction of a preformed free radical by a hydrogen atom or electron donation and is taken as a marker of antioxidant activity. The second assay involves the presence of an antioxidant during the generation of a free radical. These assays require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results. Accordingly, we have used the reduction of the water-soluble azo compound 2, 2-azo-bis(2-amidinopropane)-dihydrochloride (AAPH) and the 2,2'-cationic radical $ABTS^{\bullet+}$ decolorization assay.

The water-soluble 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) is a free radical-generating azo compound used to initiate oxidation reactions via both nucleophilic and free radical mechanisms. This model is appropriate for measuring radical-scavenging activity in

vitro because the reactivity of the peroxyl radicals by the action of AAPH shows a greater similarity to cellular activities such as lipid peroxidation. In the AAPH assay the highly reactive alkylperoxyl radicals are intercepted mainly by a hydrogen atom transfer (HAP) from the antioxidant.[22] All of our PBN derivatives studied caused inhibition of lipid peroxidation (LPO) higher than the parent PBN with only 34% of inhibition (experimental values are summarized in Table 3.1.1). FADiPBN (79%) was the most potent with value slightly higher than the reference molecule trolox (78%). This indicates that our nitrone derivatives exhibit good antioxidant potency against lipid peroxidation. In a liposome model, the superiority of Trolox over lipophilic bitailed PBN derivatives was observed, showing that nitrones do not act as chain-breaking antioxidants but rather as retarders[23] in agreement with the findings of others.[24] Although the kinetics was not studied in this work, this suggests that the DiPBN derivative exhibit a stronger retarder effect than PBN that arises from the presence of two antioxidant groups but that might also be due to the presence of a sugar group.

Concerning the ability of compounds to reduce the preformed ABTS cation radical, the majority of the tested compounds also showed very high antioxidant activity (57-100%). FADiPBN and FAPBN reduced the ABTS cation radical by 76 and 57% respectively, whereas the parent molecule PBN was by far the strongest. Comparing FADiPBN and FAPBN it seems that the presence of a second nitrone group within FAPBN leads to an increase of the antioxidant activity. The rest compounds present almost equipotent high activity. Thus, our nitrones appear to be good electron donors to the ABTS radical cation.

***In vitro* inhibition of soybean lipoxygenase.** Eicosanoids are oxygenated metabolites of arachidonic acid with a broad implication in a diversity of diseases. The lipoxygenase (LOX) catalyzes the first two steps in the metabolism of arachidonic acid to leukotrienes. LTB4 generation is considered to be important in the pathogenesis of neutrophil-mediated

inflammatory diseases[22] with a marked relation to the severity of cardiovascular diseases, asthma and cancer.

In this context, we decided to evaluate the synthesized conjugates for their ability to inhibit soybean LOX by the UV absorbance based enzyme assay.[25] Most of the LOX inhibitors are antioxidants or free radical scavengers.[26] LOXs contain a "non-heme" iron per molecule in the enzyme active site as high-spin Fe^{2+} in the native state and the high spin Fe^{3+} in the activated state. Some studies suggest a relationship between LOX inhibition and the ability of the inhibitors to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+}.[26] This inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form,[27] whereas several LOX inhibitors are excellent ligands for Fe^{3+}. NDGA, a known inhibitor of soybean LOX, has been used as a reference compound (IC_{50} 28μM). All the tested compounds inhibited LOX activity whereas the parent PBN did not present any inhibition under the reported experimental conditions. FADiPBN and Boc-[Lys(PBN)]$_2$-R_F exhibited equipotent IC_{50} values and are more potent than the other derivatives, followed by FAPBN. However their activity is much lower than the reference molecule NDGA.

	AAPH inhibition	**ABTS-• inhibition**	**LOX inhibition**
FAPBN	66 %	57 %	100 μM
FADiPBN	79 %	76 %	65 μM
Boc-Lys(PBN)-R_F	66 %	90 %	23 %
Boc-[Lys(PBN)]$_2$-R_F	52 %	100 %	65 μM
R_F-Lys(PBN)-OH	60 %	90 %	43 %
PBN	34 %	100 %	No
Trolox	78 %	91 %	-
NDGA	-	-	28μM

Table 3.1.1. Biological results of lysine-based nitrones.

III. Conclusion

On the one hand, we have designed a new amphiphilic carrier comprising lactobionolactone as a polar head, a perfluorinated hydrophobic tail and two lysines as central scaffold upon which nitrone antioxidants can be grafted. We extended that concept of divalent antioxidants for the search of a synergistic effect between nitrone PBN and antioxidant Trolox. Physical-chemical experiments have demonstrated that amphiphilic character of the carrier increase the overall lipophilicity of the antioxidants. However, the nature of the antioxidant *i.e*. hydrophile or lipophile also affects the overall amphiphilic character. More hydrophilic is the antioxidant and more lipophilic is its corresponding amphiphilic derivative. Among the divalent antioxidants synthesized, we showed that FADiPBN and FATxPBN keep their ability to trap free radicals despite the steric hindrance of the amphiphilic carrier, as it was already observed. Moreover, the slight electron-withdrawing effect brought by the amide linker also affects the electrochemical properties, leading to more hardly oxidized compounds. One the other hand, two ionic amphiphilic PBN derivatives were designed. There were found poorly water-soluble due to the small hydrophilic part compared to the fluorinated hydrophobic tail. Consequently, these two ionic PBN derivatives might be useful for targeting of the nitrone group toward hydrophobic domain such as membranes.

IV. Experimental Section. General methods and materials for the synthesis, log k'_W determination, spin-trapping and cyclic voltammetry experiments are described in chapter 2.

Synthesis of Boc-Lys(Z)-C_6F_{13} (1). Sodium azide (31.65 mmol, 2.05 g, 3 eq) was added to a solution of 1H, 1H, 2H, 2H-perfluorooctyl iodide (10.55 mmol, 5 g, 1 eq) in DMF. After 24 h of stirring at room temperature, the mixture was poured into cold water and extracted three times with Et_2O. The organic layer was washed with brine (2×), dried over Na_2SO_4, filtered, and concentrated under vacuum. The resulting yellow oil was dissolved in Et_2O with 0.60 g of 10% Pd/C and submitted to a hydrogen atmosphere for 8 h (8 bars). Filtration of the catalyst

through a pad of Celite and evaporation of the solvent gave 1H, 1H, 2H, 2H-perlfluorooctylamine (9.17 mmol, 3.33 g) in 87% yield. The resulting amino compound (8.26 mmol, 3.0 g, 1 eq), Boc-Lys(Z)-OH (9.09 mmol, 3.45 g, 1.1 eq), DCC (9.91 mmol, 2.04 g, 1.2 eq) and a catalytic amount of HOBt were dissolved in dry CH_2Cl_2 and stirred under argon atmosphere at room temperature for 5 hours, filtered and then concentrated under vacuum. Purification by flash chromatography eluted with EtOAc/CH (4:6 v/v) gave compound **1** (8.26 mmol, 4.30 g, 71 %) as a white viscous product. R_f 0.27 (EtOAc/CH 4:6 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.27 (5H, CH Ph ,m), 6.64 (1H, NH, s), 5.09 (1H, NH, d, J = 8 Hz), 5.02 (2H, CH_2Ph, s), 4.83 (1H, NH, s), 3.94 (1H, CH, s), 3.49 (2H, NH-$\underline{CH_2}$-CH_2-C_6F_{13}, q, J = 4 Hz), 3.10 (2H, $\underline{CH_2}$-NH Lys, t, J = 4 Hz), 2.19-2.32 (2H, $\underline{CH_2}$-C_6F_{13}, m), 1.40-1.87 (6H, 3 CH_2 Lys, m), 1.35 (9H, tBu, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.6, 156.0 (CO), 136.5 (C), 128.4, 128.0 (CH), 80.0 (C), 66.6 (CH_2), 54.4 (CH), 40.1, 33.9, 31.8, 31.2, 30.6 (CH_2), 28.1 (CH_3), 24.9 (CH_2); ^{19}F NMR ($CDCl_3$, 377 MHz) δ -80.8 (3F, CF_3), -114.7 (2F, CF_2), -121.9, -122.9, -123.5 (6F, 3CF_2), -126.2 (2F, CF_2).

Synthesis of Boc-Lys(Z)-Lys(Z)-C_6F_{13} (2). After removal of the tert-butoxycarbonyl group of compound **1** (2.07 mmol, 1.5 g, 1eq) using 20 % of trifluoroacetic acid in dry CH_2Cl_2, the resulting amino intermediate was concentrated under vacuum and immediately added to Boc-Lys(Z)-OH (2.69 mmol, 1.02 g, 1.3 eq), DCC (2.69 mmol, 0.55 g, 1.3 eq) and a catalytic amount of HOBt dissolved in dry CH_2Cl_2. After 5 hours of stirring under argon atmosphere at room temperature, the mixture was filtered and concentrated under vacuum. Purification by flash chromatography eluted with EtOAc/CH (6:4 v/v) and by size exclusion chromatography gave compound **2** (1.87 mmol, 1.85 g, 91 %) as a white viscous product. R_f 0.25 (EtOAc/CH 6:4 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.27 (10H, CH, m)), 6.95 (1H, NH, m), 6.54 (1H, NH, m), 5.42 (1H, NH, m), 5.02 (4H, CH_2, s), 4.95 (2H, NH, m), 4.28 (1H, CH, m), 3.92 (1H, CH, m), 3.38 (2H, CH_2, m), 3.10 (4H, CH_2, m), 2.25 (2H, CH_2, m), 1.88-1.40 (21H, CH_2 +

CH_3, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.8, 157.0, 156.7 (CO), 136.5, 136.5 (C), 128.5, 128.2, 128.1, 128.0 (CH), 80.6 (C), 66.7, 66.6 (CH_2), 53.0, 49.2 (CH), 40.3, 39.8, 33.9, 32.0, 30.8, 30.5, 29.4, 29.3 (CH_2), 28.3, 28.2, (CH_3), 25.6, 24.9, 22.5, 22.2 (CH_2). HR-MS (ESI+, m/z) calcd for $C_{41}H_{50}F_{13}N_5O_8$ [$(M+H)^+$] 988.3530, found 988.3536.

Synthesis of Fmoc-Lys(Boc)-Lys(Z)-C_6F_{13} (3). Compound **1** (2.48 mmol, 1.8 g) was dissolved in CH_2Cl_2/TFA (8:2 v/v). After 2h of stirring, the mixture was concentrated under vacuum and to give the resulting crude amino compound. A solution of Fmoc-Lys(Boc)-OH (2.98 mmol, 1.39 g, 1.2 eq), DCC (2.98 mmol, 0.62 g, 1.2 eq) and HOBt (2.98 mmol, 0.54 g, 1.2 eq) in dry CH_2Cl_2 was stirred for 30 minutes. The crude amino compound and TEA (pH=8-9) were then added to the solution and stirred under argon atmosphere for two days. The mixture was poured into NaCl, the organic layer was extracted three times with CH_2Cl_2, dried over Na_2SO_4 and concentrated under vaccum. After purification by flash chromatography eluted with EtOAc/CH (6:4), compound **3** (2.04 mmol, 2.2 g) was obtained as a white viscous powder in 82 % yield. R_f 0.45 (EtOAc/CH 7:3 v /v); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.76 (2H, CH, d, J = 4 Hz), 7.57 (2H, CH, m), 7.39 (2H, CH, t, J = 12 Hz), 7.31 (7H, CH, m), 6.91 (1H, NH, m), 6.73 (1H, NH, m), 6.71 (1H, CH, m), 5.05 (2H, CH_2, s), 5.00 (1H, NH, m), 4.72 (1H, NH, m), 4.39 (3H, CH + CH_2, m), 4.18 (1H, CH, t, J = 8 Hz), 4.11 (1H, CH, m), 3.48 (2H, CH_2, m), 3.13 (4H, $2CH_2$, m), 2.30 (2H, CH_2, m), 2.27 (2H, CH_2, m), 1.91 (2H, CH_2, m), 1.70 (2H, CH_2, m), 1.42 (11H, CH_2 + $3CH_3$, m), 1.35 (4H, $2CH_2$, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.48, 171.79, 157.23, 156.82 (CO), 143.65, 143.69, 141.28, 136.49 (C), 128.50, 128.09, 127.96, 127.78, 127.68, 127.10, 127.07, 125.00, 124.93, 120.02 (CH), 79.39 (C), 67.17, 66.63 (CH_2), 53.09, 49.33, 47.05 (CH), 40.19, 39.47, 33.77, 31.99, 30.45, 29.51, 29.25 (CH_2), 28.39, 28.28 (CH_3), 25.53, 24.86, 22.46 (CH_2); HR-MS (ESI+, m/z) calcd for $C_{48}H_{55}F_{13}N_5O_8$ [$(M+H)^+$] 1076.3843, found 1076.3832.

Synthesis of Lacto$(OAc)_8$-Lys(Z)-Lys(Z)-C_6F_{13} (4). Compound **2** (0.40 mmol, 0.40 g, 1 eq)

was dissolved in dry CH_2Cl_2/TFA (8:2, v/v) at 0°C for 2 hours and then concentrated under vacuum. In parallel, lactobionolactone (0.52 mmol, 0.18 g, 1.3 eq) was prepared according to a published procedure[4] and was added to a solution of the resulting amino derivative dissolved in EtOH with few drops of TEA (pH=8-9). The solution was stirred at 78°C for 24 h until complete consumption of the amino derivative. Then the solvent was evaporated in vacuum and the residue was added to a solution of Ac_2O/pyridine (1:1 v/v) at 0 °C. After 5 h, the mixture was poured into cold 1 N HCl and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuum. After purification by flash chromatography, eluting with EtOAc/CH (8:2) and then by size exclusion chromatography eluted with CH_2Cl_2/MeOH (1:1 v/v) compound **4** was obtained (0.29 mmol, 0.45 g, 60%) as a white powder. R_f 0.38 (EtOAc/CH 9:1 v /v) ; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34 (10H, CH, m), 7.00 (1H, NH, m), 6.91 (1H, NH, m), 6.70 (1H, NH, d, J = 4 Hz), 5.47 (1H, CH, m), 5.37 (1H, CH, d J = 2 Hz), 55.07-5.13 (7H, m), 4.99-5.02 (2H, CH, m), 4.54-4.59 (2H, CH, m), 4.00-4.08 (7H, m), 3.88 (1H, CH, t, J = 8 Hz), 3.39-3.62 (2H, CH_2, m), 3.10-3.24 (4H, CH_2, m), 2.32 (2H, CH_2, m), 1.99-2.19 (24H, CH_3, m), 1.90 (2H, CH_2, m), 1.66 (2H, CH_2, m), 1.49 (4H, CH_2, m), 1.36 (4H, CH_2, m); ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 171.85, 171.09, 170.61, 170.47, 170.04, 169.67, 169.29, 156.80 (CO), 136.51 (C), 128.53, 128.12, 128.00, 127.82 (CH), 101.59, 71.16, 70.81, 69.33, 68.93, 66.82, 66.66, 61.80, 61.03, 53.46 (CH), 40.39, 32.00, 30.49, 30.22, 29.34, 29.17 (CH_2), 20.70, 20.57 (CH_3); HR-MS (ESI+, m/z) calcd for $C_{64}H_{79}F_{13}N_5O2_5$ [$(M+H)^+$] 1564.4856, found 1564.4891.

Synthesis of Lacto(OAc)$_8$-Lys(Boc)-Lys(Z)-C$_6$F$_{13}$ (5). Compound **3** (1.39 mmol, 1.5 g) was dissolved in ACN/DEA (9:1 v/v) for 1 hour and then concentrated under vacuum. In parallel, lactobionolactone (1.81 mmol, 0.62 g, 1.3 eq) was prepared according to a published procedure[4] and was added to a solution of the resulting amino derivative dissolved in EtOH with few drops of TEA (pH=8-9). The solution was stirred at 60°C for 48 h and the solvent

was evaporated in vacuum. The residue was then added to a solution of Ac_2O/pyridine (1:1 v/v) at 0 °C. After 5 h, the mixture was poured into cold 1 N HCl and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuum. After purification by flash chromatography, eluting with EtOAc/CH (8:2 v/v) and by size exclusion chromatography eluted with CH_2Cl_2/MeOH (1:1 v/v) compound **5** was obtained (0.83 mmol, 1.27 g, 62%) as a white powder. R_f 0.33 (EtOAc/CH 8:2 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (5H, CH, m), 7.10 (1H, NH, m), 6.97 (1H, NH, m), 6.90 (1H, NH, m), 5.48 (1H, CH, m), 5.38 (1H, CH, m), 5.31 (1H, CH, m), 4.93-5.29 (7H, CH + CH_2 + NH, m), 4.52-4.61 (2H, CH, m), 4.18-4.32 (4H, CH + CH_2, m), 4.10 (2H, CH2, m), 3.91 (1H, CH, m), 3.38-3.61 (2H, CH_2, m), 3.02-3.22 (4H, CH_2, m), 2.37 (2H, CH_2, m), 1.99-2.21 (24H, CH_3, m), 1.34-1.90 (21H, CH_2 + CH_3, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.82, 171.19, 170.62, 170.56, 170.44, 170.15, 170.00, 169.63, 169.26, 156.92, 156.70, 156.33 (CO), 136.57 (C), 128.49, 128.06, 127.95, 101.51 (CH), 79.14 (C), 78.01, 72.22, 71.14, 70.82, 70.27, 69.40, 69.01, 66.83 (CH), 66.59, 61.82, 61.02 (CH_2), 54.09, 53.50 (CH), 40.40, 39.98, 33.87, 31.96, 30.38, 29.32 (CH_2), 28.39 (CH_3), 25.58, 24.89, 22.92, 22.52 (CH_2), 20.68, 20.56, 20.46 (CH_3); HR-MS (ESI+, m/z) calcd for $C_{61}H_{81}F_{13}N_5O2_5$ [$(M+H)^+$] 1530.5013, found 1530.5011.

Synthesis of Lacto(OAc)$_8$-Lys(PBN)-Lys(PBN)-C$_6$F$_{13}$ (6). At 0°C, compound **4** (0.26 mmol, 0.40 g, 1eq) was dissolved in ethanol/acetic acid (99:1 v/v) and catalytic amount of 10% Pd/C was slowly added. The reaction mixture was submitted to a hydrogen atmosphere for 18 h (5 bars). After filtration of the catalyst through a pad of Celite and evaporation of the solvent under vacuum, the resulting amino compound was added to a solution of HOSu-PBN (0.57 mmol, 0.22 g, 2.2 eq) in dry CH_2Cl_2 at room temperature and under argon. After 24 h, the solvent was removed under vacuum and the crude mixture was purified by flash chromatography eluting with EtOAc/MeOH (95:5 v/v) and by size exclusion chromatography

eluting with CH_2Cl_2/MeOH (1:1 v/v) to give the acetylated derivative **6** (0.12 mmol, 0.20 g, 46% yield) as a white powder. R_f 0.30 (EtOAc/MeOH 95:5 v /v); ^{1}H NMR (MeOD, 400 MHz) δ 8.31 (4H, CH, m), 7.89 (1H, CH, s), 7.88 (1H, CH, s), 7.82 (4H, CH, m), 5.41 (1H, CH, d, J = 4 Hz), 5.27 (1H, CH, d, J = 2 Hz), 5.19 (1H, CH, d, J = 2 Hz), 4.97-5.05 (3H, CH, m), 4.68 (1H, CH, d, J = 2 Hz), 4.40 (1H, CH, m), 3.91-4.18 (7H, m), 3.38 (6H, CH_2, m), 2.31 (2H, CH_2, m), 1.98-1.84 (24H, CH_3, m), 1.74 (2H, CH_2, m), 1.64 (2H, CH_2, m), 1.50 (22H, CH_2 + CH_3, m), 1.36 (4H, CH_2, m); ^{13}C NMR (MeOD, 100 MHz) δ 174.40, 173.83, 172.22, 172.17, 172.14, 171.80, 171.51, 171.38, 171.27, 170.45, 169.32, 169.23 (CO), 137.27, 134.86 (C), 133.44, 133.37, 130.51, 130.47, 128.46, 128.38 (CH), 102.72, 79.88, 74.27, 72.71, 72.68, 72.39, 72.13, 71.11, 70.80, 70.54, 68.77, 63.09, 62.60, 54.89 (CH), 40.96, 40.56, 32.78, 32.32, 32.02, 31.29, 29.98, 29.93 (CH_2), 28.42 (CH_3), 24.41, 24.05 (CH_2), 20.97, 20.91, 20.81, 20.72, 20.61, 20.48 (CH_3); HR-MS (ESI+, m/z) calcd for $C_{72}H_{93}F_{13}N_7O_{25}$ [$(M+H)^+$] 1702.6013, found 1702.6036.

Synthesis of Lacto(OAc)$_8$-Lys(Trolox)-Lys(Z)-C$_6$F$_{13}$ (8).Compound **5** (0.39 mmol, 0.60 g, 1 eq) was dissolved in dry CH_2Cl_2/TFA (8:2, v/v) at 0°C for 2 hours and then concentrated under vacuum. The resulting amino derivative was dissolved in dry CH_2Cl_2 with few drops of DIEA (pH=8-9) and was added to a solution of Trolox® (0.51 mmol, 0.13 g, 1.3 eq), DCC (0.51 mmol, 0.11 g, 1.2 eq) and a catalytic amount of HOBt in dry CH_2Cl_2. After one night of stirring under argon atmosphere at room temperature, the solution was filtered and then concentrated under vacuum. Purification by flash chromatography eluted with EtOAc/CH (8:2 v/v) and then by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1:1 v/v) gave compound **8** (0.29 mmol, 0.48 g, 74 %) as a white powder. R_f 0.22 (EtOAc/CH 9:1 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.33 (5H, CH, m), 6.89 (2H, NH, m), 6.79 (1H, NH, m), 6.48 (1H, NH, m), 6.36 (1H, NH, m), 5.46 (1H, CH, m), 5.38 (1H, CH, d, J = 4 Hz), 5.29-5.18 (2H, CH, m), 5.08-5.12 (4H, CH + CH_2, m), 5.02 (1H, CH, m), 4.51-4.61 (2H, CH_2, m), 4.30

(1H, CH, m), 4.21 (2H, CH_2, m), 4.02-4.10 (2H, CH_2, m), 3.90 (2H, CH_2, m), 3.44-3.65 (2H, CH_2, m), 3.05-3.30 (4H, CH_2, m), 2.33-2.63 (6H, CH_2, m), 1.99-2.20 (33H, CH_3, m), 1.76-1.84 (2H, CH_2, m), 1.70-1.47 (9H, CH_3 + CH_2, m), 1.38 (2H, CH_2, m), 1.21 (2H, CH_2, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.87, 172.16, 172.07, 171.53, 171.42, 170.74, 170.67, 170.28, 170.20, 170.15, 169.80, 169.44, 169.09 (CO), 145.80, 144.69, 136.69 (C), 128.67, 128.25, 128.11 (CH), 122.97, 121.89, 120.28, 118.52, 118.29 (C), 101.76, 78.67 (CH), 72.52, 72.38, 71.32, 71.25, 70.99, 70.48, 69.17 (CH), 67.02, 66.96, 66.81, 61.98, 61.11, 60.54 (CH_2), 54.42, 53.60, 40.69, 38.26, 38.06, 32.15, 30.70, 29.73, 29.43, 25.46, 25.30 (CH_2) 20.90, 20.87, 20.75, 20.72, 20.66, 12.51, 12.05, 11.99, 11.55 (CH_3). HR-MS (ESI+, m/z) calcd for $C_{70}H_{89}F_{13}N_5O_{26}$ [$(M+H)^+$] 1662.5588, found 1662.5581.

Synthesis of Lacto(OAc)$_8$-Lys(Trolox)-Lys(PBN)-C6F13 (9). At 0°C, compound **8** (0.26 mmol, 0.44 g, 1eq) was dissolved in ethanol/acetic acid (99:1 v/v) and catalytic amount of 10% $Pd/_C$ was slowly added. The reaction mixture was submitted to a hydrogen atmosphere for 18 h (5 bars). After filtration of the catalyst through a pad of Celite and evaporation of the solvent under vacuum, the resulting amino compound was added to a solution of HOSu-PBN (0.31 mmol, 0.12 g, 1.2 eq) in dry CH_2Cl_2 at room temperature and under argon. After 24 h, the solvent was removed under vacuum and the crude mixture was purified by flash chromatography eluting with EtOAc/MeOH (95:5 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1:1 v/v) to give the acetylated derivative **9** (0.15 mmol, 0.26 g, 58% yield) as a white powder. R_f 0.24 (EtOAc/MeOH 95:5 v /v) ; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.28 (2H, CH, d, J = 8 Hz), 7.80 (2H, CH, d, J = 12 Hz), 7.62 (1H, CH, s), 7.27 (2H, NH, m), 7.09 (2H, NH, m), 6.37 (1H, NH, m), 6.32 (1H, NH, m), 5.49 (1H, CH, m), 5.34 (2H, CH, m), 5.07-5.16 (2H, CH, m), 5.01 (1H, CH, m), 4.60 (1H, CH, m), 4.45 (1H, CH, m), 4.28 (1H,CH, m), 4.18 (2H, CH_2, m), 4.05 (2H, CH_2, m), 3.90 (2H, CH_2, m), 3.46-3.55 (4H, CH_2, m), 3.27 (2H, CH_2, m), 2.35-2.51 (6H, CH_2, m), 1.96-2.17 (33H, CH_3, m), 1.74 (2H, CH_2, m),

1.59 (12H, CH_3 + CH_2, m), 1.49 (2H, CH_2, m), 1.38 (2H, CH_2, m), 1.24 (4H, CH_2, m), 1.07 (2H, CH_2, m); ^{13}C NMR (CDl_3, 100 MHz) δ 174.61, 174.51, 172.19, 172.10, 171.74, 171.63, 170.46, 170.02, 169.63, 169.25, 168.68, 168.58, 167.12 (CO), 145.69, 144.51, 135.15, 133.68 (C), 129.31, 128.68, 127.07 (CH), 123.41, 122.88, 121.57, 120.68, 117.67 (C), 101.56, 78.38 (CH), 78.27 (C), 72.45, 71.43, 70.84, 70.24, 69.31, 69.24, 66.81 (CH), 61.69, 60.86 (CH_2), 54.21, 54.11, 53.50 (CH), 39.36, 38.20, 38.02, 31.86, 30.45, 29.62, 29.55, 28.84 (CH_2), 28.24 (CH_3), 25.23, 25.09, 2307 (CH_2), 20.69, 20.64, 20.61, 20.53, 20.45, 12.41, 11.82, 11.74, 11.44 (CH_3). HR-MS (ESI+, m/z) calcd for $C_{74}H_{96}F_{13}N_6O_{26}$ [$(M+H)^+$] 1731.6166, found 1731.6161.

Synthesis of Lacto(OH)$_8$-Lys(PBN)-Lys(PBN)-C$_6$F$_{13}$ (FADiPBN, 7). A catalytic amount of sodium methoxide was added under argon to a solution of compound **6** (0.10 mmol, 0.17 g, 1 eq) in MeOH. The mixture was stirred for 4 h, and few drops of 1 N HCl solution were added to neutralize the solution. Purification by size exclusion chromatography eluting with MeOH give FADiPBN (0.09 mmol, 0.12 g, 86% yield). R_f 0.53 (EtOAc/MeOH/H_2O 7:2:1 v/v/v); ^{1}H NMR (MeOD, 400 MHz) δ 8.41 (4H, CH, d, J = 4 Hz), 7.98 (2H, CH, s), 7.90 (4H, CH, m), 4.51 (1H, CH, d, J = 4 Hz), 4.44 (1H, CH, d, J = 2 Hz), 4.34 (1H, CH, m), 4.25 (2H, CH, m), 3.99 (1H, CH, t, J = 6 Hz), 3.90 (1H, CH, m), 3.84-4.72 (5H, m), 3.62-3.58 (3H, CH, m), 3.55-3.362.31 (6H, CH_2, m), 2.49-2.36 (2H, CH_2, m), 1.93-1.45 (30H, CH_2 + CH_3, m); ^{13}C NMR (MeOD, 100 MHz) δ 176.17, 174.42, 174.36, 169.33, 169.31 (CO), 137.35, 137.30, 134.78, 134.74 (C), 133.66, 133.58, 130.57, 130.56, 128.41, 128.38, 105.87, 83.15, 77.31, 74.82, 74.47, 73.24, 73.13, 72.77, 72.69, 70.30, 63.72, 62.66, 58.34, 55.15, 55.01 (CH), 40.70, 4.060, 32.80, 32.28, 32.18, 31.50, 31.29, 31.07 (CH_2), 28.42 (CH_3), 25.44, 24.40, 22.02, 18.39 (CH_2); HR-MS (ESI+, m/z) calc for $C_{56}H_{77}F_{13}N_7O_{17}$ [$(M+H)^+$] 1366.5168, found 1366.5166.

Synthesis of Lacto(OH)$_8$-Lys(Trolox)-Lys(PBN)-C$_6$F$_{13}$ (FATxPBN, 10). A catalytic amount of sodium methoxide was added under argon to a solution of compound **9** (0.13

mmol, 0.22 g, 1 eq) in MeOH. The mixture was stirred for 5 h, and few drops of 1 N HCl solution were added to neutralize the solution. Purification by size exclusion chromatography eluting with MeOH give FATxPBN (0.12 mmol, 0.17 g, 96% yield). R_f 0.60 (EtOAc/MeOH/H_2O 7:2:1 v/v/v); ^{1}H NMR (MeOD, 400 MHz) δ 8.36 (1H, OH, s), 8.30 (2H, CH, d, J = 8 Hz), 7.88 (1H, CH, s), 7.82 (2H, CH, d, J = 8 Hz), 4.44 (1H, CH, d, J = 8 Hz), 4.34 (1H, CH, s), 4.05-4.18 (3H, CH, m), 3.91 (1H, CH, m), 3.81 (1H, CH, m), 3.64-3.75 (5H, CH + CH_2, m), 3.21-3.52 (7H, CH + CH_2, m), 2.90-3.16 (2H, CH_2, m), 2.20-2.50 (6H, CH_2, m), 2.05 (6H, CH_3, m), 1.96 (3H, CH_3, m), 1.50-2.06 (16H, CH_2 + CH_3, m), 1.38 (4H, CH_2, m), 1.16-1.20 (4H, CH_2, m); ^{13}C NMR (MeOD, 100 MHz) δ 176.60, 176.57, 175.93, 174.34, 170.49, 169.21 (CO), 147.05, 145.75, 137.12, 134.73 (C), 133.31, 130.45, 128.34 (CH), 124.97, 122.88, 122.85, 122.48, 118.72 (C), 105.92, 83.41 (CH), 79.12 (C), 77.17, 74.71, 74.33, 73.07, 72.73, 72.61, 70.27 (CH), 63.54, 62.59, 61.48 (CH_2), 55.06, 54.88 (CH), 40.46, 39.65, 32.73, 32.34, 32.12, 31.25, 30.65, 29.92, 28.40, 25.01 (CH_2), 21.62 (CH_3), 14.44, 12.91, 12.18, 11.95 (CH_2). HR-MS (ESI+, m/z) calc for $C_{56}H_{77}F_{13}N_7O_{17}$ [$(M+H)^+$] 1395.5322, found 1395.5314.

Synthesis of C_6F_{13}-Lys(Z)-OtBu (<u>11</u>). 2H,2H,3H,3H-perfluorononanoic acid (2.55 mmol, 1g, 1eq), DCC (3.31 mmol, 683 mg, 1.3 eq) and a catalytic amount of HOBt were dissolved in dry CH_2Cl_2. After few minutes of activation, H_2N-Lys(Z)-OtBu (2.81 mmol, 1g, 1.1 eq) and DIEA (pH = 8-9) were added and the mixture was stirred 16h under argon atmosphere.The solution was then filtered, concentrated under vacuum and purified by flash chromatography eluted with EtOAc/CH (3:7) to give compound **<u>11</u>** (2.41 mmol, 1.71 g, 90 %)as a white powder. R_f 0.28 (EtOAc/CH 3:7 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31 (5H, CH, m), 6.29 (1H, NH, m),5.10 (2H, CH_2, s), 4.83 (1H, NH, m), 4.45 (1H, CH, q, J = 20 Hz), 3.19 (2H, CH_2, q, J = 16 Hz), 2.52 (4H, CH_2, m), 1.85-1.24 (15H, m); ^{13}C NMR ($CDCl_3$, 100 MHz)δ171.42, 169.57, 156.61 (CO), 136.52 (C), 128.51, 128.11, 128.00 (CH), 82.36, 66.63

(C), 52.63 (CH), 31.90, 29.49 (CH_2), 27.96 (CH_3), 26.88, 26.63, 21.99 (CH_2).^{19}F NMR ($CDCl_3$, 377 MHz)δ -80.80 (3F, CF_3), -114.55 (2F, CF_2), -121.90, -122.88, -123.47 (6F, $3CF_2$), -126.14 (2F, CF_2). HR-MS (ESI+, m/z) calcd for $C_{27}H_{32}F_{13}N_2O_5$ [$(M+H)^+$] 711.2104, found 711.2101.

Synthesis of C_6F_{13}-Lys(PBN)-OtBu (12). Compound **11** (1.34 mmol, 1g, 1eq) was dissolved in ethanol and 0.080 g of 10% Pd/C was slowly added at 0 °C. The reaction mixture was then submitted to a 5 bar hydrogen atmosphere during 2 h. After filtration of the catalyst through a pad of Celite and concentration under vacuum, the resulting amino compoundwas added to a solution of HOSu-PBN (1.61 mmol, 612 mg, 1.2 eq) in dry CH_2Cl_2 at room temperature under argon atmosphere. After 14h the solution was concentrated under vacuum and purified by flash chromatography eluted with EtOAc/CH (8:2) to give compound **12** as a white powder (0.94 mmol, 734 mg) in 74 % yield. R_f 0.22 (EtOAc/CH 8:2 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.32 (2H, CH, d), 7.82 (2H, CH, d), 7.26 (1H, CH, s), 6.41 (2H, NH, m), 4.45 (1H, CH, q, J = 20 Hz), 3.47 (2H, CH_2, m), 2.48 (4H, CH_2, m), 1.88-1.62 (15H, m), 1.49 (9H, CH_3, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ171.40, 169.80, 167.05(CO), 135.15, 133.71 (C), 129.01, 128.60, 126.99 (CH), 82.20, 71.41 (C), 52.67 (CH), 39.26, 31.75, 29.05 (CH_2), 28.26, 27.94 (CH_3), 26.80, 26.66, 22.24 (CH_2); ^{19}F NMR ($CDCl_3$, 377 MHz) δ -80.77 (3F, CF_3), -114.56 (2F, CF_2), -121.90, -122.86, -123.48 (6F, $3CF_2$), -126.13 (2F, CF_2). MS ESI$^+$ $[M+H]^+$=780.2; HR-MS (ESI+, m/z) calcd for $C_{31}H_{39}F_{13}N_3O_5$ [$(M+H)^+$] calc 780.2682, found 780.2686.

Synthesis of C_6F_{13}-Lys(PBN)-OH (13). At 0°C, compound **12** (0.38 mmol, 0.30 g, 1eq) was dissolved in a solution of dry CH_2Cl_2 / TFA (5:5 v/v) and 150µL of scavenger TES was added to the mixture. The solution was stirred 3h under argon atmosphere and was concentrated under vacuum. After purification by flash chromatography eluted with EtOAc/MeOH (8:2 v/v) containing 1% of AcOH, compound **13** was obtained (0.29 mmol, 0.212 g) as a white powder in 76% yield. R_f 0.18 (EtOAc/MeOH 8:2 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.58

(1H, NH, m), 8.41 (2H, CH, d), 7.98 (1H, CH, s), 7.89 (2H, CH, d), 4.39 (1H, CH, m), 3.40 (2H, CH_2, q, J = 12 Hz), 2.56 (4H, 2 CH_2, m), 1.95-1.48 (15H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 176.81, 173.87, 170.83 (CO), 138.50, 135.66 (C), 134.52, 131.76, 129.71 (CH), 73.99 (C), 54.97 (CH), 42.05, 33.60, 31.28 (CH_2), 30.18 (CH_3), 29.09, 28.87, 25.46 (CH_2); ^{19}F NMR ($CDCl_3$, 377 MHz) δ -79.06 (3F, CF_3), -112.74 (2F, CF_2), -119.90, -120.88, -121.56 (6F, $3CF_2$), -124.22 (2F, CF_2). MS ESI$^+$ $[M+H]^+$ = 724.1; HR-MS (ESI+, m/z) calcd for $C_{27}H_{32}F_{13}N_2O_5$ $[(M+H)^+]$ 724.2056, found 724.2058.

Synthesis of Boc-Lys(PBN)-C_6F_{13} (<u>14</u>).At 0°C, compound **<u>1</u>** (1.38 mmol, 1 g, 1eq) was dissolved in diethyl ether and 0.08 g of 10% Pd/C was slowly added. The reaction mixture was submitted to a hydrogen atmosphere for 30 h (7 bars). After filtration of the catalyst through a pad of Celite and evaporation of the solvent under vacuum, the resulting amino compound was added to a solution of HOSu-PBN (1.52 mmol, 0.58 g, 1.1eq) in dry CH_2Cl_2 at room temperature under argon. After 24 h, the solvent was removed under vacuum and the crude mixture was purified by flash chromatography eluting with EtOAc/CH (9:1 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1:1 v/v) to give compound **<u>14</u>** (0.0.90 mmol, 0.72 g, 69% yield) as a white powder. R_f 0 .22 (EtOAc/CH 8:2 v /v); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.33 (2H, CH, d, J = 4 Hz), 7.83 (2H, CH, d, J = 4 Hz), 7.61 (1H, CH, s), 6.89 (1H, NH, t, J = 6 Hz), 6.63 (1H, NH, m), 5.31 (1H, NH d, J = 4 Hz), 4.06 (1H, CH, m), 3.76 (2H, CH_2, m), 3.44 (2H, CH_2, m), 2.35 (2H, CH_2, m), 1.87 (1H, m), 1.62 (12H, m), 1.42 (11H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.58, 167.10 (CO), 135.19, 133.71 (C), 129.09, 128.65, 127.04 (CH), 80.25, 71.45 (C), 54.31 (CH), 39.09, 31.86, 30.87, 30.66, 29.06 (CH_2), 28.30, 28.23 (CH_3), 22.53 (CH_2). HR-MS (ESI+, m/z) calcd for $C_{27}H_{32}F_{13}N_2O_5$ $[(M+H)^+]$ 795.2791, found 795.2794.

Synthesis of H_2N-Lys(PBN)-C_6F_{13} (<u>15</u>). At 0°C, compound **<u>14</u>** (0.13 mmol, 0.10 g, 1eq) was dissolved in a solution of dry CH_2Cl_2 / TFA (8:2 v/v) and 40µL of scavenger TES were added

to the mixture. The solution was stirred 2h under argon atmosphere and was concentrated under vacuum. After purification by silica gel chromatography eluted with EtOAc/MeOH (9:1 v/v), compound **15** was obtained (0.01 mmol, 0.07 g) as a white powder in 78% yield. R_f 0.18 (EtOAc/MeOH 8:2 v /v); ^{1}H NMR (MeOD, 400 MHz) δ 8.41 (2H, CH, d, J = 8 Hz), 7.99 (1H, CH, s), 7.89 (2H, CH, d, J = 8 Hz), 3.75 (1H, CH, t, J = 12 Hz), 3.56 (2H, CH_2, t, J = 12 Hz), 3.41 (2H, CH_2, t, J = 8 Hz), 2.45 (2H, m), 1.86 (2H, m), 1.69 (2H, m), 1.66 (9H, s), 1.47 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.38 (CO), 137.12, 134.87 (C), 133.50, 130.51, 128.28 (CH), 72.72 (C), 54.55 (CH), 40.36, 32.99, 32.72, 30.14 (CH_2), 28.39 (CH_3), 27.66, 23.36 (CH_2); MS ESI$^+$ $[M+H]^+$ = 724.1; HR-MS (ESI+, m/z) calcd for $C_{26}H_{32}F_{13}N_4O_3$ $[(M+H)^+]$ 695.2267, found 695.2269.

Synthesis of C_6F_{13}-Lys(Z)-Lys(Z)-OtBu (16).The *tert*-butoxycarbonyl protecting group of compound **11** (0.62 mmol, 0.44 g, 1eq) was remonved using 50 % of trifluoroacetic acid in dry CH_2Cl_2, the resulting compound was concentrated under vacuum and added to a solution of H-Lys(Z)-OtBu (0.68 mmol, 0.25 g, 1.1 eq), DCC (0.79 mmol, 0.16 g, 1.3 eq) and a catalytic amount of HOBt in dry CH_2Cl_2, few drops of TEA were added to control pH~8-9. After one night of stirring under argon atmosphere and at room temperature, the mixture was filtered and concentrated under vacuum. The crude product was purify by flash chromatography eluted with EtOAc/CH (4:6 v/v) to give compound **16** (0.26 mmol, 0.26 g, 44 %) as a white product. R_f 0.36 (EtOAc/CH 6:4 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30 (10H, CH, m), 7.13 (1H, NH, m), 7.06 (1H, NH, m), 5.40 (1H, NH, m), 5.30 (1H, NH, m), 5.07 (4H, CH_2, m), 4.55 (1H, CH, m), 4.36 (1H, CH, m), 3.14 (4H, CH_2, m), 2.45 (4H, CH_2, m), 1.78 (2H, CH_2, m), 1.66 (2H, CH_2, m), 1.34-1.50 (17H, CH_2 + CH_3, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.72, 171.21, 171.15, 170.30, 156.75 (CO), 136.60, 136.56 (C), 128.47, 128.45, 128.07, 128.02 (CH), 82.12 (C), 66.62, 66.53 (CH_2), 53.01, 52.81 (CH), 40.27, 40.19, 32.00, 31.35, 29.26 (CH_2), 27.87 (CH_3), 26.72, 26.51, 26.29, 22.16, 22.09

(CH_2). HR-MS (ESI+, m/z) calcd for $C_{41}H_{50}F_{13}N_4O_8$ [$(M+H)^+$] 973.3421, found 973.3429.

Synthesis of C_6F_{13}-Lys(PBN)-Lys(PBN)-OtBu (17). At 0°C, compound **16** (0.51 mmol, 0.50 g, 1eq) was dissolved in ethanol with a catalytic amount of 10% $Pd/_C$ and the mixture was submitted, overnight, to a hydrogen atmosphere (6 bars). After filtration of the catalyst through a pad of Celite and evaporation of the solvent under vacuum, the resulting amino compound was added to a solution of HOSu-PBN (1.12 mmol, 0.38 g, 2.2 eq) in dry CH_2Cl_2 at room temperature under argon atmosphere. After 24 h, the solvent was removed under vacuum and the crude mixture was purified by flash chromatography eluting with EtOAc/MeOH (95:5 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1:1 v/v) to give compound **17** (0.30 mmol, 0.34 g, 60% yield) as a white powder. R_f 0.32 (EtOAc/MeOH 9:1 v /v) ; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.29 (4H, CH, m), 7.84 (4H, CH, m), 7.59 (2H, CH, m), 7.05 (3H, NH, m), 4.40 (2H, CH, m), 3.33-3.55 (4H, CH_2, m), 2.43 (4H, CH_2, m), 1.73-1.85 (39H, CH_2 + CH_3, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.63, 171.22, 170.35, 167.32, 167.14 (CO), 135.32, 135.16, 133.61, 135.58 (C), 129.25, 129.15, 128.65, 128.62, 128.50, 127.25, 127.15, 127.08 (CH), 82.16, 71.40 (C), 53.31, 52.58 (CH), 39.19, 31.41, 28.86, 28.73 (CH_2), 28.28, 27.95 (CH_3), 26.71, 26.61, 22.32, 22.11 (CH_2). HR-MS (ESI+, m/z) calcd for $C_{49}H_{64}F_{13}N_6O_8$ [$(M+H)^+$] 1111.4578, found 1111.4584.

Synthesis of Boc-Lys(PBN)-Lys(PBN)-C_6F_{13} (18).Compound **2** (0.50 mmol, 0.50 g, 1eq) was dissolved in ethanol at 0°C with a catalytic amount of 10% Pd/C. The mixture was then submitted, overnight, to a hydrogen atmosphere (6 bars). After filtration of the catalyst through a pad of Celite and evaporation of the solvent under vacuum, the resulting amino compound was added to a solution of HOSu-PBN (1.11 mmol, 0.42 g, 2.2 eq) in dry CH_2Cl_2 at room temperature and under argon atmposphere. After 24 h, the solvent was removed under vacuum and the crude mixture was purified by flash chromatography eluting with EtOAc/MeOH (95:5 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH

(1:1 v/v) to give compound **18** (0.26 mmol, 0.29 g, 50% yield) as a white powder. R_f 0.28 (EtOAc/MeOH 9:1 v /v) ; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.30 (4H, CH, m), 7.88 (4H, CH, m), 7.64 (2H, CH, m), 7.40 (2H, NH, m)), 7.27 (1H, NH, m), 7.08 (1H, NH, m), 5.75 (1H, NH, m), 4.35 (1H, CH, m), 3.97 (1H, CH, m), 3.51 (4H, CH_2, m), 3.31 (3H, CH_2 + NH, m), 2.35 (2H, CH_2, m), 1.31-2.05 (39H, CH_2 + CH_3, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.09, 172.14, 171.19, 167.40, 167.20 (CO), 156.64, 135.55, 135.30, 133.51, 133.46 (C), 129.70, 129.64, 128.83, 127.27, 127.17 (CH), 80.48, 71.49, 71.43 (C), 55.58, 53.20 (CH), 50.67, 39.09, 38.98, 31.94, 30.49, 29.67, 29.02, 28.86 (CH_2), 28.25, 28.24 (CH_3), 22.57, 22.45 (CH_2). HR-MS (ESI+, m/z) calcd for $C_{49}H_{65}F_{13}N_7O_8$ [$(M+H)^+$] 1126.4687, found 1126.4680.

^{19}F NMR experiments. Critical micelle concentrations were determined using ^{19}F NMR spectroscopy. All the samples were dissolved in a deuterated water/water mixture (1:9, v/v) containing CF_3CO_2Na as internal reference. A concentrated solution of 1-3 mM was prepared and a range of diluted solutions were obtained by successive dilutions. All solutions were vortexed for a few minutes and then incubated at room temperature for 12 h. ^{19}F NMR spectra were recorded at 25°C on a Bruker AC 400 spectrometer operating at 400 MHz. The NMR spectrum was calibrated using the internal reference peak of CF_3CONa and chemical shift of the CF_3 group was given in ppm.

Dynamic light scattering. Hydrodynamic particle size distributions were determined on a Zetasizer Nano-S model 1600 (Malvern Instruments, UK) equipped with a He-Ne laser (λ = 633 nm, 4.0 mW). The surfactant solution was prepared 24 h prior to measurements using Milli-Q water. The solution was vortexed for a few minutes and then incubated at room temperature for 24 h. The surfactant solution was passed through a 0.45-µm filter before being transferred into a 45-µL low-volume quartz batch cuvette. The time-dependent correlation function of the scattered light intensity was measured at an angle of 173^o (backscattering detection). The hydrodynamic diameter (D_H) of the particles was estimated

from their diffusion coefficient (D) using the Stokes–Einstein equation, $D = k_B T / 3\pi\eta D_H$, where k_B is the Boltzmann's constant, T absolute temperature, and η the viscosity of the solvent. CONTIN analysis was used for evaluating autocorrelation functions. All measurements were done at (25 ± 0.5)°C.

Inhibition of linoleic acid lipid peroxidation.[27] Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234 nm. AAPH is used as a free radical initiator. Ten microliters of the 16 mM linoleic acid sodium salt solution was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 µL of 40 mM AAPH solution. Oxidation was carried out in the presence of 10 µL of the compounds.

$ABTS^{\bullet+}$ – decolorization assay in ethanolic solution for antioxidant activity.[28] ABTS is dissolved in water to a 2 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) is produced by reacting the ABTS stock solution with 0.17 mM potassium persulfate in phosphate buffer (pH 7.4, 20 mM) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For steady state measurements, 100 mM $ABTS^{\bullet+}$ was used. For the present study, the 100 mM $ABTS^{\bullet}$ solution (200 µL) was diluted with ethanol (790 µL) to an absorbance of 0.70 at 734 nm, equilibrated at room temperature, mixed with 10 µL of the tested compounds (stock solutions 10 mM) and the absorbance reading was taken at room temperature 1 min after the initial mixing. Trolox was used as a standard.

Soybean LOX inhibition study *in vitro*.[27] The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 ml) and 0.2 ml of enzyme solution (1/9 x 10^{-4} w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234nm was recorded and compared with the appropriate standard inhibitor NDGA.

Chapter III

Towards the improvement of the Bioavailability of Nitrones

Chapter III – Part 2

Melanoma-targeting of α-Phenyl-*N-tert*-butyl Nitrones

I. Introduction

Melanoma is a very aggressive skin cancer which has dramatically increased in recent years and has become a major worldwide public health problem.[29] It represents only a minority of skin cancers but it is the worst of them and appears in any part of the body. Melanoma is a result of an uncontrolled growth of melanocytes, the cells responsible for the production of melanin, which are found in the basal layer of the epidermis. More precisely, melanin is produced in highly specialized organelles known as melanosomes. The life span of patients with metastases at stage IV is less than one year[30, 31] and unfortunately, there is currently no specific and effective treatment for this cancer. The lack of treatments and specific therapies led researchers to develop compounds with a specific affinity to melanoma tissues. Different melanoma targets have been investigated such as melanin pigment which can be itself a specific target for melanoma tissue. Melanin is made of covalently linked eumelanin and pheomelanin (Figure 3.2.1) but the exact chemical structure of melanin is still under debate.[32]

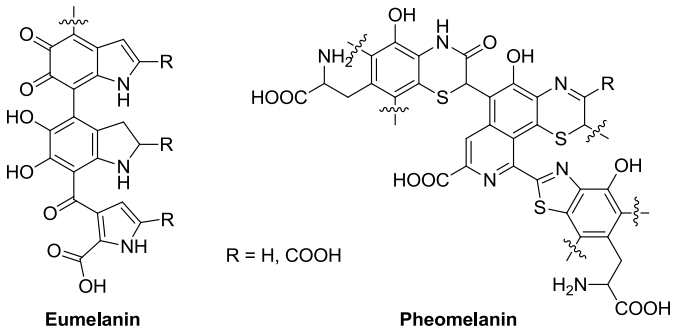


Figure 3.2.1. Structures of eumelanin and pheomelanin.

Benzamide derivatives used to specifically target the melanoma. Many drugs derived from polycyclic aromatic compounds are able to bind to melanin, probably due to the interaction between the aromatic rings of the drugs and the aromatic rings of the melanin subunits.[33] A wide range of benzamide derivatives such as spermidine benzamide derivatives,[34] iodinated

benzamides derivatives[35, 36] and aromatic or heteroaromatic benzamide analogs[33] were developed as potent melanoma-seeking agents.

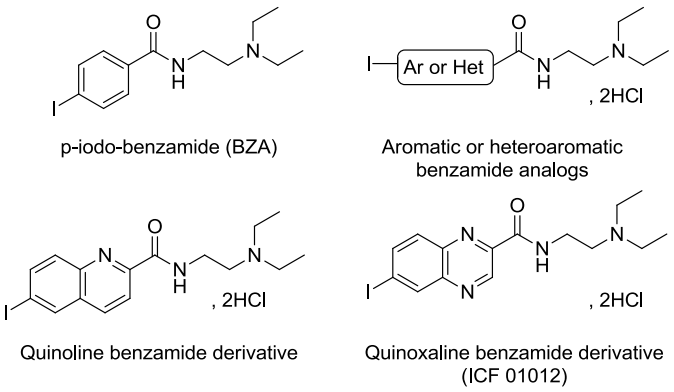


Figure 3.2.2. General structures of benzamide derivatives with a high affinity for melanin. (Ar or Het = naphtalene, pyridine, indole, benzo[*b*]furan, benzo[*b*]thiophene, imidazole[1,2-a]pyridine, benzimidazole, quinoline, quinolone, isoquinoline, quinoxaline, 1,6-naphthyridine).[33]

All benzamides derivatives represented above (Figure 3.2.2) exhibit an affinity for melanoma but with different pharmacokinetic profiles; the quinoline and quinoxaline derivatives were the most potents.[33] After pharmacomodulation study, ICF 01012 was selected for its high, sustained and specific tumor concentration with a rapid clearance from non-target organs, making it promising for application in targeted radionuclide therapy.[37] A convenient analytical protocol, based on high-performance liquid chromatographic method, was developed for detection of [^{131}I] ICF 01012 in biological samples in order to follow the *in vivo* metabolism.[38] Anti-tumoral study of [^{131}I] ICF01012 showed a strong efficacy associated with low toxicity, supporting the concept of targeted radionuclide therapy using [^{131}I] radiolabelled iodoquinoxaline for an effective melanoma treatment.[39]

The high affinity of ICF 01012 towards melanin led Chezal and co-workers to develop three melanin-targeted ligands in order to graft goldonium based nanoparticules that have been developed for multimodal imaging and theranostic applications. The binding site of the three compounds is a free amino function and in two of them a pegylated spacer was added in one

or other of the two extremities in order to study the importance of both ionic and hydrophobic sites.[40]

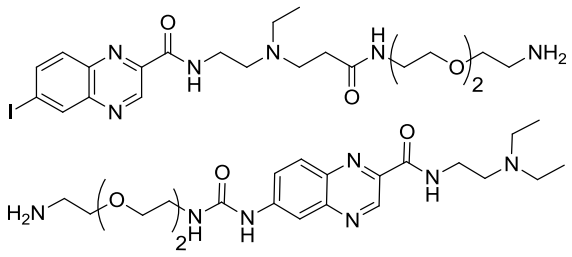


Figure 3.2.3. Structure of melanin-targeted ligands with pegylated spacer.

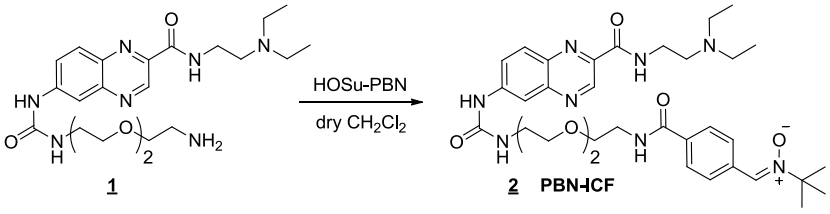
Macular pigment and melanin in age-related maculopathy: Targeting of nitrones. Tumor cells produce high amounts of ROS and melanocytes are continuously exposed to reactive biochemical species. This is finely tuned via the intrinsic antioxidant and radical properties of melanin. But, an imbalance in the antioxidant system can lead to endogenous generation of ROS in human melanomas and melanin is able to induce DNA damage. Moreover, the macular pigment and melanin have been suggested to protect against age-related maculopathy (ARM) by its abilities to scavenge free radicals and PBN has already shown potency against light-induced retinal degeneration.[41] Therefore, the presence of a PBN moiety able to scavenge radicals conjugated with a melanoma-targeting ligand could be beneficial against ARM.

A melanin-targeted ligand grafted on the PBN will ensure intracellular targeting of the nitrone group specifically into the melanomas. In collaboration with the University of Clermont-Ferrand, we developed two nitrone derivatives conjugated with a benzamide compound susceptible to target the melanoma. This would allow us to target PBN in melanoma tissues in which reactive species are widely formed. In order to achieve this targeting, we synthesized two linear nitrones conjugated to the melanin-targeted ligand via an amide bond. Chezal and col. have provided compound **1**, a ICF01012 derivative in which iodine was replaced by a pegylated spacer.[40] The first derivative denoted PBN-ICF (compound **2**) was directly

obtained by conjugating PBN to the ICF group, while the second derivative bearing a perfluorocarbonated chain was obtained by grafting the R_F-Lys(PBN)-OH derivative onto ICF, leading to compound **3** also called R_F-Lys(PBN)-ICF.

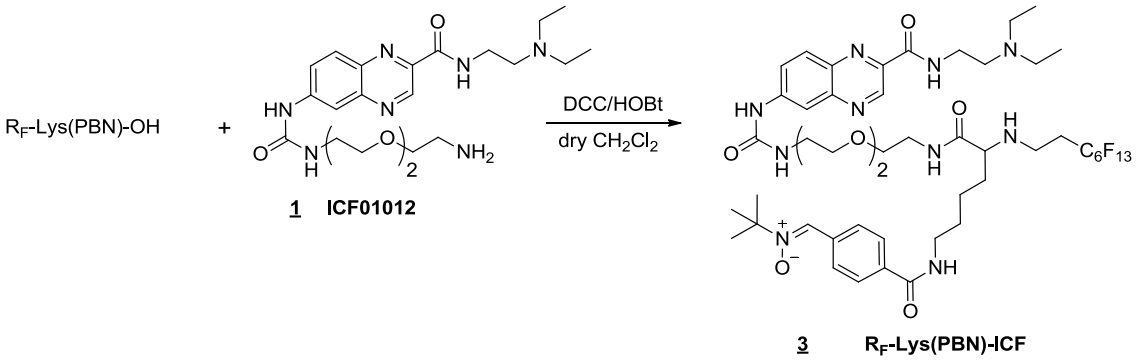
II. Results and discussion

Synthesis of PBN-ICF (2). The primary amino group of the melanin targeted ligand was linked in a single step with HOSu-PBN, in dry dichloromethane. The mixture was stirred at room temperature and under argon atmosphere until complete consumption of the amine (t=6 h). The solvent was then evaporated and purification on silica gel chromatography followed by size exclusion chromatography led to PBN-ICF (**2**) in 97 % yield.



Scheme 3.2.1. Synthesis of a melanoma-targeted nitrone (PBN-ICF).

Synthesis of R_F-Lys(PBN)-ICF (3). In continuation with the project described in the first part of this chapter, an amphiphilic lysine-based compound targeting melanoma was synthesized. Compound **1** was used as a hydrophilic part and R_F-Lys(PBN)-OH as a hydrophobic part. R_F-Lys(PBN)-OH was previously synthesized following procedure describes in the first part of this chapter. The amino group of compound **1** was then coupled to the acidic function of R_F-Lys(PBN)-OH using dicyclohexylcarbodiidimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. After complete consumption of the amino compound, reaction was stopped. Purification on silica gel chromatography followed by size exclusion chromatography, gave R_F-Lys(PBN)-ICF in 60 % yield.

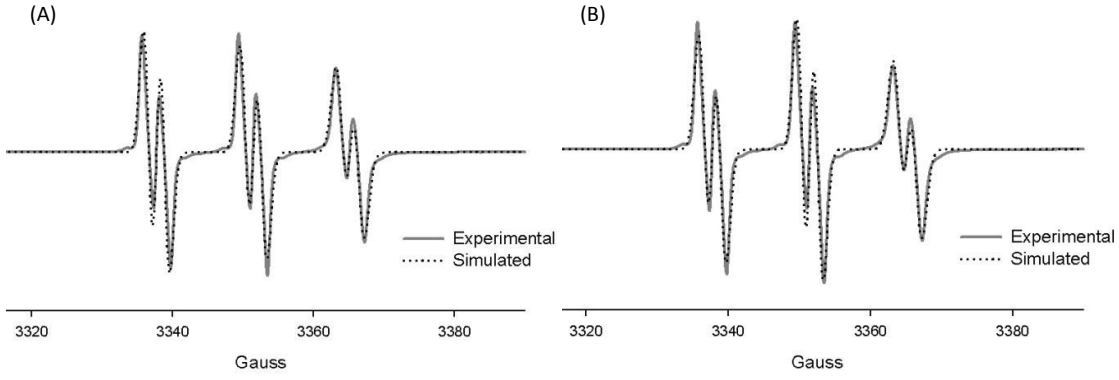


Scheme 3.2.2. Synthesis of the amphiphilic melanoma-targeted nitrone R_F-Lys(PBN)-ICF.

Physicochemical Measurements.

The PBN-ICF compound was soluble in water until ~ 4 $g.L^{-1}$, whereas its corresponding amphiphilic derivative was more hardly soluble due to the presence of the fluorinated chain. We found that R_F-Lys(PBN)-ICF was soluble up to ~1 $g.L^{-1}$ afterward the solution became slightly cloudy.

Electronic Paramagnetic Resonance (EPR) spectroscopy. The spin-trapping properties of the two melanoma-targeted derivatives were studied using Electron Paramagnetic Resonance (EPR) spectroscopy to evaluate their spin strapping ability. We investigated the spin-adducts formation of methoxy radical ($CH_3O^{\bullet}$) and superoxide radical ($O_2^{\bullet}$), using two standard systems conditions DMSO/MeOH/$Pb(OAc)_4$ and H_2O_2/Pyridine, respectively.



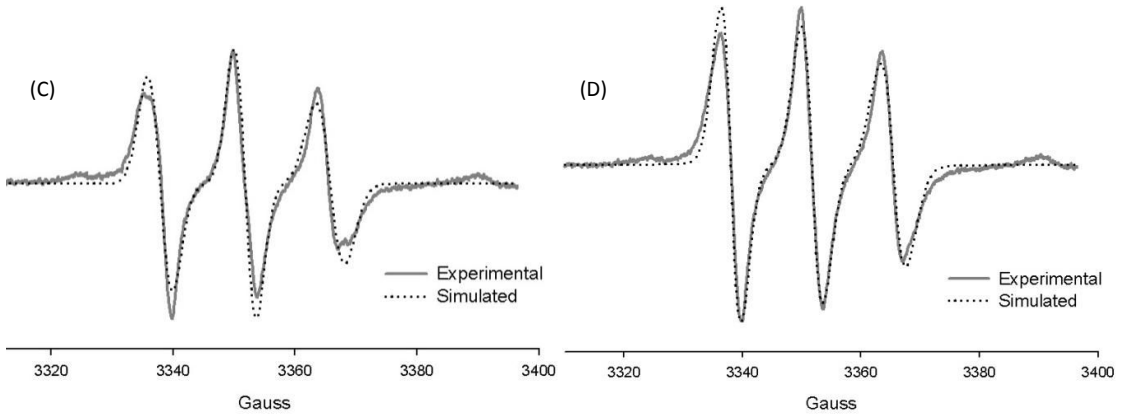


Figure 3.2.4. Experimental and simulated EPR spectrum of methoxy radical adduct of (A) PBN-ICF and (B) R_F-Lys(PBN)-ICF; experimental and simulated EPR spectrums of superoxide radical adduct of (C) PBN-ICF and (D) R_F-Lys(PBN)-ICF.

Both nitrone derivatives were found to trap the methoxy and superoxide radical. A standard six lines spectrum was observed for the methoxy spin-adduct formation, corresponding to the hyperfine coupling with the nitrogen and the hydrogen in β position of the nitrone function. After simulation, we obtained values of a_N = 13.73 G and a_H = 2.23 G for PBN-ICF and a_N = 13.72 G and a_H = 2.23 G for R_F-Lys(PBN)-ICF, in good agreement with a PBN-type radical adduct. Despite of the bulky ICF-group grafted, PBN keeps its ability to trap free radicals, as previously observed for amphiphilic nitrones. The asymmetric signals observed for both PBN-ICF and R_F-Lys(PBN)-ICF are characteristics of anisotropic conditions leading to a slow molecular tumbling motion. This suggests that even compound **2** has a slightly amphiphilic character, in which PBN plays the role of hydrophobic group. Concerning the superoxide radical trapping, only three lines were observed for both compounds. This might be due to the presence of oxygen in the pyridine solution, leading to broader lines and therefore the interactions with β-hydrogen cannot be determined. For the hyperfine coupling with the nitrogen, we found constant values of 13.92 G for compound **2** and 13.70 for compound **3**.

Cyclic voltammetry (CV). The electrochemical characterization of the two melanoma-targeted nitrones was studied using cyclic voltammetry (CV) in aqueous conditions

containing 50 mM of NaCl. In these conditions, the nitronyl function of PBN moiety exhibited an irreversible one-step reduction with a cathodic peak potential about -1.74 V vs. Ag/AgCl, in agreement with values of the literature.[19] Both PBN-ICF and R_F-Lys(PBN)-ICF are more easily reduced than PBN, with potentials values of -1.45 V and -1.38 V, respectively (Figure 3.2.5). The electron-withdrawing amide bond in *para*-position of the PBN moiety is responsible of the highest reductive potentials observed in water for the two melanoma-targeted nitrone derivatives. A second peak potential was observed at about ~ -1.0 V, this peak was also present in the voltammogram of ICF alone and was therefore assigned to the melanoma-targeted ligand.

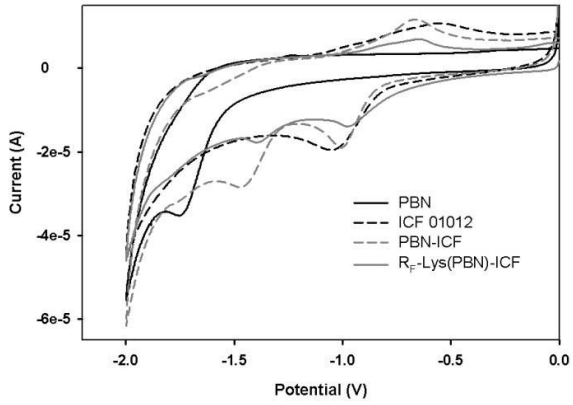


Figure 3.2.5. Reduction of PBN, ICF01012, PBN-ICF and R_F-Lys(PBN)-ICF in 50 mM NaCl at 0.1 V/s.

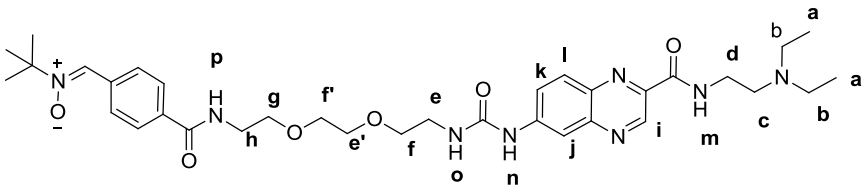
III. Conclusion

Two new conjugates of a melanoma-targeting ligand and a PBN moiety were synthesized. EPR experiments have demonstrated that despite the presence of a bulky group, the nitronyl function is still able to trap free radicals. As observed for amphiphilic derivatives described in part 1 of this chapter, cyclic voltammetry experiments have demonstrated that the electron-withdrawing effect of the amide bond that links the PBN to the carriers tend to decrease the reduction potential of compounds **2** and **3** in aqueous media. Biological evaluation against

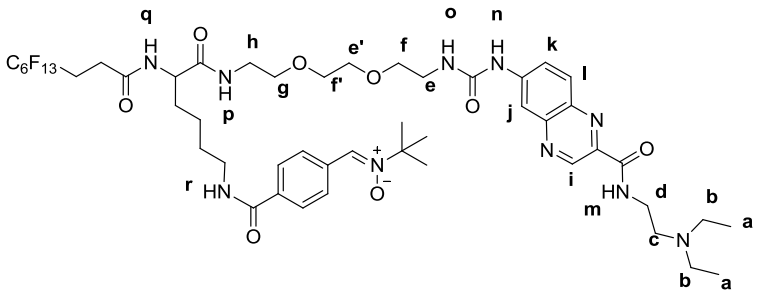
macular degenerative disorders is currently in progress, for these two melanoma-targeted derivatives, in the lab of Pr Isabelle Ranchon-Cole in Clermont-Ferrand.

IV. Experimental Section

General methods and materials for the synthesis, spin-trapping experiments and cyclic voltammetry experiments are described in part 1 of chapter 2.



Synthesis of PBN-ICF (2). Under agron atmosphr, compound **1**[40] (0.17 mmol, 80 mg, 1 eq) was dissolved in dry CH_2Cl_2. HOSu-PBN (0.21 mmol, 70 mg, 1.2 eq) was added to the solution. After 22 h of stirring at room temperature, the solvent was evaporated under vacuum. The crude mixture was purified by silica gel chromatography eluted with CH_2Cl_2/EtOH (6:4 v/v) and then by size exclusion chromatography eluted with CH_2Cl_2/MeOH (1:1 v/v) to give compound **2** (0.17 mmol, 111 mg, 97% yield) as a yellow powder. R_f = 0.18 (CH_2Cl_2/EtOH 6:4 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.35 (1H, H(i), s), 9.03 (1H, NH(n), s), 8.37 (1H, NH(p), s), 8.17 (2H, CH Ph, d, *J* = 8 Hz), 7.99 (1H, CH = N^+, s), 7.91 (1H, H(l), d, *J* = 12 Hz), 7.76 (3H, CH Arom. (k), m), 7.53 (1H, NH(o), s), 7.49 (1H, H(j), s), 6.23 (1H, NH(m), s), 3.62 (2H, H(h), t, *J* = 4 Hz), 3.53-3.58 (8H, H(e, e', f, f"), m), 3.46 (2H, H(c), m), 3.33 (2H, H(d), t, *J* = 8 Hz), 2.72 (2H, H(g), t, *J* = 8 Hz), 2.6 (4H, H(b), q, *J* = 8 Hz), 1.5 (9H, tBu, s), 1.03 (6H, H(a), t, *J* = 8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.8, 163.9, 155.6 (CO), 144.8, 143.6 (C), 143.5 (CH), 141.1, 136.4, 135.2, 133.5 (C), 129.9, 129.7, 128.7, 127.2, 124.5, 113.2 (CH), 71.4 (C), 70.5, 70.2, 70.0, 69.6, 51.5, 47.0, 40.1, 39.6, 36.9 (CH_2), 28.2, 11.4 (CH_3). HR-MS (ESI+, m/z) calcd for $C_{27}H_{32}F_{13}N_2O_5$ [$(M+H)^+$] 665.3775, found 665.3735.



Synthesis of R_F-Lys(PBN)-ICF (3). R_F-Lys(PBN)-OH (0.047 mmol, 34 mg, 1.1 eq), DCC (0.06 mmol, 110 mg, 1.1 eq) and a catalytic amount of HOBt were dissolved in dry CH_2Cl_2. Compound **1** (0.04 mmol, 20 mg, 1 eq) was added and the mixture was stirred overnight under argon atmosphere. The solution was then filtered over a pad of Celite, concentrated under vacuum and purified by silica gel chromatography eluted with CH_2Cl_2/EtOH (6:4 v/v) and asize exclusion chromatography eluted with CH_2Cl_2 / MeOH (1:1 v/v) to give compound **3** (0.03 mmol, 30 mg, 60% yield) as a yellow powder. R_f 0.18 (CH_2Cl_2/EtOH 6:4 v /v); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.36 (1H, H(i), s), 9.03 (1H, NH(n), s), 8.38 (1H, NH(p), s, 8.17 (2H, CH Arom., d, J = 8 Hz), 8.02 (1H, H(l), d, J = 12 Hz), 7.94 (1H, CH = N^+, s), 7.80 (1H, NH(r), s), 7.75 (2H, CH Arom., d, J = 8 Hz), 7.56 (1H, NH(o), s), 7.50 (2H, H(j)-H(k), m), 7.42 (1H, NH (q), s), 6.30 (1H, NH(m), s), 4.36 (1H, CH*, s), 3.28-3.55 (16H, H(c, d, e, f, e', f', g, h), m), 2.71 (2H, $\underline{CH_2}$-NH Lys, t, J = 12 Hz), 2.62 (4H, H(b), q, J = 8 Hz), 2.35-2.49 (4H, $\underline{CH_2}$-$\underline{CH_2}$-C_6F_{13}, m), 1.67-1.75 (2H, CH_2 Lys, m), 1.51 (11H, tBu- CH_2 Lys, s), 1.31-1.35 (2H, CH_2 Lys, m), 1.02 (6H, H(a), t, J = 8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.3, 170.9, 167.5, 163.8, 155.6 (CO), 144.7, 143.7 (C), 143.6 (CH), 141.3, 136.5, 135.0, 133.6 (C), 130.1, 129.3, 128.6, 127.1, 124.4, 113.1 (CH), 71.4 (C), 70.3, 70.2, 69.9, 69.4 (CH_2), 53.6 (CH), 51.5, 47.0 (CH_2), 39.4 ($\underline{CH_2}$-C_6F_{13}, J = 20 Hz), 36.9, 31.3 (CH_2), 28.2 (CH_3), 26.7, 26.5, 26.3, 22.5 (CH_2), 11.4 (CH_3); ^{19}F NMR ($CDCl_3$, 377 MHz) δ -80.8 (3F, CF_3), -114.7 (2F, CF_2), -121.9, -122.9, -123.5 (6F, $3CF_2$), -126.2 (2F, CF_2). MS ESI$^+$ $[M+H]^+$ = 1167.5; HR-MS (ESI+, m/z) calcd for $C_{27}H_{32}F_{13}N_2O_5$ $[(M+H)^+]$ 1167.4701, found 1167.4696.

References

[1] G. Durand, B. Poeggeler, S. Ortial, A. Polidori, F.A. Villamena, J. Böker, R. Hardeland, M.A. Pappolla, B. Pucci, Amphiphilic amide nitrones: A new class of protective agents acting as modifiers of mitochondrial metabolism, J. Med. Chem., 53 (2010) 4849-4861.

[2] G. Durand, A. Polidori, O. Ouari, P. Tordo, V. Geromel, P. Rustin, B. Pucci, Synthesis and preliminary biological evaluations of ionic and nonionic amphiphilic alpha-phenyl-N-tert-butylnitrone derivatives, J. Med. Chem., 46 (2003) 5230-5237.

[3] G. Durand, R.A. Prosak, Y. Han, S. Ortial, A. Rockenbauer, B. Pucci, F.A. Villamena, Spin trapping and cytoprotective properties of fluorinated amphiphilic carrier conjugates of cyclic versus linear nitrones, Chem. Res. Toxicol., 22 (2009) 1570-1581.

[4] S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M.A. Pappolla, J. Boeker, R. Hardeland, B. Pucci, Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents, J. Med. Chem., 49 (2006) 2812-2820.

[5] B. Meunier, Hybrid Molecules with a Dual Mode of Action: Dream or Reality?†, Accounts of Chemical Research, 41 (2007) 69-77.

[6] F. Belluti, G. Fontana, L.D. Bo, N. Carenini, C. Giommarelli, F. Zunino, Design, synthesis and anticancer activities of stilbene-coumarin hybrid compounds: Identification of novel proapoptotic agents, Bioorganic & Medicinal Chemistry, 18 (2010) 3543-3550.

[7] M. Koufaki, A. Detsi, Design and Synthesis of Antioxidant α-Lipoic Acid Hybrids, in: D. Armstrong (Ed.) Advanced Protocols in Oxidative Stress II, vol. 594, Humana Press, 2010, pp. 297-309.

[8] J.J. Harnett, M. Auguet, I. Viossat, C. Dolo, D. Bigg, P.-E. Chabrier, Novel lipoic acid analogues that inhibit nitric oxide synthase, Bioorganic & Medicinal Chemistry Letters, 12 (2002) 1439-1442.

[9] S.R. Venkatachalam, A. Salaskar, A. Chattopadhyay, A. Barik, B. Mishra, R. Gangabhagirathi, K.I. Priyadarsini, Synthesis, pulse radiolysis, and in vitro radioprotection studies of melatoninolipoamide, a novel conjugate of melatonin and α-lipoic acid, Bioorganic & Medicinal Chemistry, 14 (2006) 6414-6419.

[10] M. Koufaki, A. Detsi, E. Theodorou, C. Kiziridi, T. Calogeropoulou, A. Vassilopoulos, A.P. Kourounakis, E. Rekka, P.N. Kourounakis, C. Gaitanaki, P. Papazafiri, Synthesis of chroman analogues of lipoic acid and evaluation of their activity against reperfusion arrhythmias, Bioorganic & Medicinal Chemistry, 12 (2004) 4835-4841.

[11] S. Menichetti, M.C. Aversa, F. Cimino, A. Contini, C. Viglianisi, A. Tomaino, Synthesis and "double-faced" antioxidant activity of polyhydroxylated 4-thiaflavans, Organic & Biomolecular Chemistry, 3 (2005) 3066-3072.

[12] J. Yang, G.-Y. Liu, D.-L. Lu, F. Dai, Y.-P. Qian, X.-L. Jin, B. Zhou, Hybrid-Increased Radical-Scavenging Activity of Resveratrol Derivatives by Incorporating a Chroman Moiety of Vitamin E, Chemistry – A European Journal, 16 (2010) 12808-12813.

[13] D. Hadjipavlou-Litina, G.E. Magoulas, S.E. Bariamis, D. Drainas, K. Avgoustakis, D. Papaioannou, Does conjugation of antioxidants improve their antioxidative/anti-inflammatory potential?, Bioorganic & Medicinal Chemistry, 18 (2010) 8204-8217.

[14] G. Durand, A. Polidori, J.P. Salles, M. Prost, P. Durand, B. Pucci, Synthesis and antioxidant efficiency of a new amphiphilic spin-trap derived from PBN and lipoic acid, Bioorganic & Medicinal Chemistry Letters, 13 (2003) 2673-2676.

[15] G.T. Balogh, K. Vukics, Á. Könczöl, Á. Kis-Varga, A. Gere, J. Fischer, Nitrone derivatives of trolox as neuroprotective agents, Bioorganic & Medicinal Chemistry Letters, 15 (2005) 3012-3015.

[16] H.P.-M. Atamna, A., B.N. Ames, N-t-butylhydroxylamine, a hydrolysis product of a-phenyl-N-t-butyl nitrone, is more potent in delaying senescence in human lung fibroblasts, J. Biol. Chem., 275 (2000) 6741-6748.

[17] W. Chamulitrat, S.J. Jordan, R.P. Mason, K. Saito, R.G. Cutler, Nitric oxide formation during light-induced decomposition of phenyl N-tert-butylnitrone, Journal of Biological Chemistry, 268 (1993) 11520-11527.

[18] P. Barthélémy, V. Tomao, J. Selb, Y. Chaudier, B. Pucci, Fluorocarbon−Hydrocarbon Nonionic Surfactants Mixtures: A Study of Their Miscibility, Langmuir, 18 (2002) 2557-2563.

[19] G.L. McIntire, H.N. Blount, H.J. Stronks, R.V. Shetty, E.G. Janzen, Spin Trapping in Electrochemistry. 2. Aqueous and Nonaqueous Electrochemical Characterization of Spin Traps, J. Phys. Chem., 84 (1980) 916-921.

[20] B. Tuccio, P. Bianco, J.-C. Bouteiller, P. Tordo, Electrochemical characterisation of b-phosphorylated nitrone spin traps, Electrochimica Acta, 44 (1999) 4631-4634.

[21] D. Huang, B. Ou, R.L. Prior, The Chemistry behind Antioxidant Capacity Assays, Journal of Agricultural and Food Chemistry, 53 (2005) 1841-1856.

[22] S.W. Crooks, R.A. Stockley, Leukotriene B4, The International Journal of Biochemistry & Cell Biology, 30 (1998) 173-178.

[23] S. Ortial, S. Morandat, M. Bortolato, B. Roux, A. Polidori, B. Pucci, G. Durand, PBN derived amphiphilic spin-traps. II/Study of their antioxidant properties in biomimetic membranes, Coll. Surf. B, 113 (2014) 384– 393.

[24] L.R.C. Barclay, M.R. Vinqvist, Do spin traps also act as classical chain-breaking antioxidants? A quantitative kinetic study of phenyl-*tert*-butylnitrone (PBN) in solution and in liposomes, Free Radical Biol. Med., 28 (2000) 1079-1090.

[25] I.B. Taraporewala, J.M. Kauffman, Synthesis and structure–activity relationships of anti-inflammatory 9, 10-dihydro-9-oxo-2-acridine-alkanoic acids and 4-(2-carboxyphenyl)aminobenzenealkanoic acids, Journal of Pharmaceutical Sciences, 79 (1990) 173-178.

[26] K. Muller, 5-Lipoxygenase and 12-lipoxygenase: attractive targets for the development of novel antipsoriatic drugs, Arch. Pharm. , 327 (1994) 3-19.

[27] D. Hadjipavlou-Litina, T. Garnelis, C.M. Athanassopoulos, D. Papaioannou, Kukoamine A analogs with lipoxygenase inhibitory activity, Journal of Enzyme Inhibition and Medicinal Chemistry, 24 (2009) 1188-1193.

[28] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine, 26 (1999) 1231-1237.

[29] J.F. Thompson, R.A. Scolyer, R.F. Kefford, Cutaneous melanoma, The Lancet, 365 (2005) 687-701.

[30] C.M. Balch, A.C. Buzaid, S.-J. Soong, M.B. Atkins, N. Cascinelli, D.G. Coit, I.D. Fleming, J.E. Gershenwald, A. Houghton, J.M. Kirkwood, K.M. McMasters, M.F. Mihm, D.L. Morton, D.S. Reintgen, M.I. Ross, A. Sober, J.A. Thompson, J.F. Thompson, Final Version of the American Joint Committee on Cancer Staging System for Cutaneous Melanoma, Journal of Clinical Oncology, 19 (2001) 3635-3648.

[31] C.M. Balch, S.-J. Soong, J.E. Gershenwald, J.F. Thompson, D.S. Reintgen, N. Cascinelli, M. Urist, K.M. McMasters, M.I. Ross, J.M. Kirkwood, M.B. Atkins, J.A. Thompson, D.G. Coit, D. Byrd, R. Desmond, Y. Zhang, P.-Y. Liu, G.H. Lyman, A. Morabito, Prognostic Factors Analysis of 17,600 Melanoma Patients: Validation of the American Joint Committee on Cancer Melanoma Staging System, Journal of Clinical Oncology, 19 (2001) 3622-3634.

[32] K. Shanmuganathan, J.H. Cho, P. Iyer, S. Baranowitz, C.J. Ellison, Thermooxidative Stabilization of Polymers Using Natural and Synthetic Melanins, Macromolecules, 44 (2011) 9499-9507.

[33] J.M. Chezal, J. Papon, P. Labarre, C. Lartigue, M.J. Galmier, C. Decombat, O. Chavignon, J. Maublant, J.C. Teulade, J.C. Madelmont, N. Moins, Evaluation of radiolabelled (hetero)aromatic analogues of -(2-diethylaminoethyl)-4-iodobenzamide for imaging and targeted radionuclide therapy of melanoma, J. Med. Chem., 51 (2008) 3133-3144.

[34] M.-F. Moreau, J. Papon, P. Labarre, N. Moins, M.l. Borel, M. Bayle, B. Bouchon, J.-C. Madelmont, Synthesis, in vitro binding and biodistribution in B16 melanoma-bearing mice of new iodine-125 spermidine benzamide derivatives, Nuclear Medicine and Biology, 32 (2005) 377-384.

[35] T.Q. Pham, I. Greguric, X. Liu, P. Berghofer, P. Ballantyne, J. Chapman, F. Mattner, B. Dikic, T. Jackson, C. Loc, A. Katsifis, Synthesis and Evaluation of Novel Radioiodinated Benzamides for Malignant Melanoma, Journal of Medicinal Chemistry, 50 (2007) 3561-3572.

[36] N. Moins, J. Papon, H.l.n. Seguin, D. Gardette, M.-F. Moreau, P. Labarre, M. Bayle, J. Michelot, J.-C. Gramain, J.-C. Madelmont, A. Veyre, Synthesis, characterization and comparative biodistribution study of a new series of p-Iodine-125 benzamides as potential melanoma imaging agents, Nuclear Medicine and Biology, 28 (2001) 799-808.

[37] J.C. Madelmont, J.M. Chezal, O. Chavignon, J.C. Teulade, N. Moins, Labelled analogues of halobenzamides as radiopharmaceuticals, in, WO2008012782.

[38] D. Denoyer, P. Labarre, J. Papon, E. Miot-Noirault, M.-J. Galmier, J.-C. Madelmont, J.-M. Chezal, N. Moins, Development of a high-performance liquid chromatographic method for the determination of a new potent radioiodinated melanoma imaging and therapeutic agent, Journal of Chromatography B, 875 (2008) 411-418.

[39] M. Bonnet-Duquennoy, J. Papon, F. Mishellany, P. Labarre, J.L. Guerquin-Kern, T.D. Wu, M. Gardette, J. Maublant, F. Penault-Llorca, E. Miot-Noirault, A. Cayre, J.C. Madelmont, J.M. Chezal, N. Moins, Targeted radionuclide therapy of melanoma : Anti-tumoural efficacy studies of a new (131)I labelled potential agent, Int. J. Cancer, 125 (2009) 708-716.

[40] J. Morlieras, J.-M. Chezal, E. Miot-Noirault, A. Roux, L. Heinrich-Balard, R. Cohen, S. Tarrit, C. Truillet, A. Mignot, R. Hachani, D. Kryza, R. Antoine, P. Dugourd, P. Perriat, M. Janier, L. Sancey, F. Lux, O. Tillement, Development of gadolinium based nanoparticles having an affinity towards melanin, Nanoscale, 5 (2013) 1603-1615.

[41] I. Ranchon, S. Chen, K. Alvarez, R.E. Anderson, Systemic Administration of Phenyl-N-tert-Butylnitrone Protects the Retina from Light Damage, Investigative Ophthalmology & Visual Science, 42 (2001) 1375-1379.

CONCLUSION

Les travaux entrepris au cours de cette Thèse avaient pour but d'améliorer les propriétés de piégeage de radicaux libres ainsi que les propriétés antioxydantes de la PBN. Notre premier objectif a été de modifier la structure de base de la PBN par ajout de différents substituants afin d'améliorer ses propriétés intrinsèques. Par la suite, nous avons essayé de favoriser son transport au sein des fluides biologiques ainsi que son passage cellulaire, en greffant la PBN sur des structures possédant un motif de ciblage spécifique. L'objectif de cette seconde partie était de disposer, à long terme, d'outils plus efficaces dans le traitement de pathologies associées au stress oxydant.

Dans un premier temps, nous avons développé plusieurs analogues simples de la PBN afin de moduler ses propriétés intrinsèques. Nous nous sommes d'abord focalisés sur des modifications de la partie *N*-*tert*-butyl que nous avons substituée par des groupements aux propriétés électroniques différentes. Des analogues mono, di et tri-substitués ont également été développés. Les études de calcul de densités de charge ont quant à elles été effectuées sur une plus large gamme de composés. Les résultats ont montré une augmentation de la charge positive du carbone de la nitrone en fonction de l'ajout de substituants sur la partie *N*-*tert*-butyl. Des études de cinétique de piégeage de spin, par UV visible ou par spectroscopie RPE, ont démontré une addition nucléophile du radical superoxyde et du radical phényl sur le carbone de la nitrone. La détermination des propriétés redox de nos composés par voltammétrie cyclique nous a permis de montrer que la présence de groupements électro-attracteurs sur la partie *N*-*tert*-butyl rend l'oxydation de la fonction nitrone plus difficile. De plus, l'évaluation de l'activité cytoprotectrice *in-vitro* de nos nitrones contre le peroxyde d'hydrogène nous a permis de mettre en évidence une corrélation avec les propriétés électrochimiques. Nous avons remarqué que les nitrones à faible potentiel d'oxydation

protégeaient mieux les cellules contre un stress oxydant induit par du peroxyde d'hydrogène. Ceci démontre que la nature des substituants en β du nitronyl a une influence directe sur les propriétés de la fonction nitrone, au travers des effets inductifs. Dans la série que nous avons développée, la nitrone substituée par une liaison amide (PBN-CH_2NHAc) s'est montrée la plus intéressante avec un faible potentiel d'oxydation, de bonnes capacités de piégeage et une bonne activité antioxydante contre le peroxyde d'hydrogène.

Sur une série de dérivés de PBN substitués en *para* du phényl par des groupements aux effets électroniques variables, des travaux préliminaires avaient démontré l'influence importante des effets mésomères de ces substituants en *para*. Au cours de cette thèse, nous avons donc étudié leurs propriétés électrochimiques. Nos expériences de voltammétrie cyclique nous ont permis de montrer une corrélation entre les constantes de Hammett des substituants et les potentiels redox des nitrones. Les composés substitués par des groupements électro-donneurs se sont avérés plus facilement oxydés et plus difficilement réduits tandis que l'inverse a été constaté pour les composés substitués par des groupements électro-attracteurs. Ces informations nous ont conduits à suggérer que l'effet mésomère des groupements électro-donneurs tend à stabiliser le nitroxyde cationique intermédiaire, favorisant ainsi l'oxydation. Parmi les composés étudiés, ceux substitués par un groupe diméthyl-amine ou par un méthoxy ont montré de meilleures propriétés électrochimiques, avec de faibles potentiels d'oxydation. De plus, ces études de cinétique de piégeage par RPE ont montré que tous les dérivés piégeaient mieux le radical phényl que la PBN. D'après les calculs de densité de charge et les expériences de piégeage de radicaux libres effectuées sur les deux séries de composés, il semblerait que l'addition du radical phényl sur la nitrone soit plutôt sous contrôle orbitalaire que sous contrôle de charge. Finalement, le composé substitué par un methoxy s'est montré le plus efficace de cette série, avec un faible potentiel d'oxydation et de bonnes propriétés de

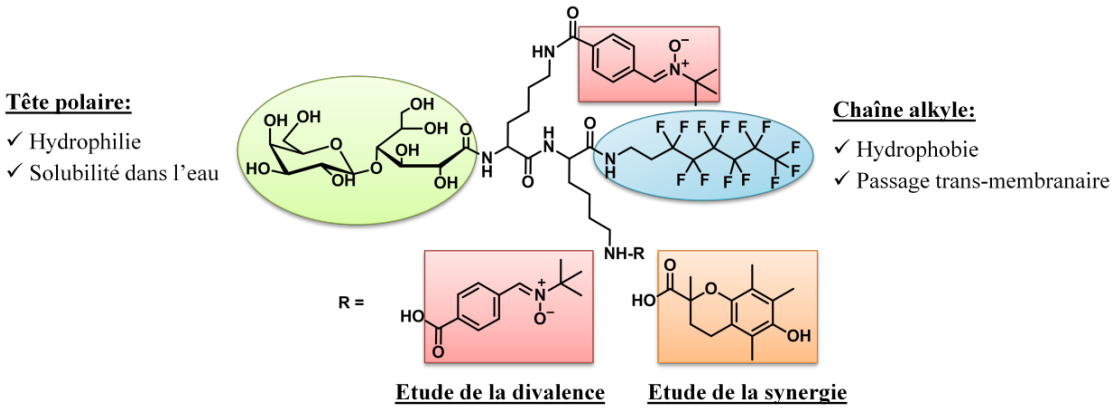
piégeage, faisant de la liaison éther un bras de liaison intéressant pour d'éventuelles fonctionnalisations en para du phényl de la PBN.

Un troisième type de modulation a été effectué par substitution du *tert*-butyl par un groupement cyclohexane. Cette modification avait pour but de rigidifier la molécule et donc de lui conférer une meilleure stabilité des adduits formés. Les études de voltammétrie cyclique ont montré que l'ajout du cycle n'avait pas d'influence significative sur les propriétés redox de la nitrone. Par contre, les dérivés conservent leur capacité à piéger les radicaux libres et les adduits de spin semblent plus intenses et plus stables que ceux de la PBN. Des études de cinétiques sont actuellement en cours pour vérifier cette hypothèse.

Aux vues des résultats obtenus avec ces trois types de modifications et dans le but d'améliorer davantage les propriétés intrinsèques de la PBN, il serait désormais opportun de synthétiser une série de composés modifiés à la fois en *para* du phényl et sur la partie *N*-*tert*-butyl de la nitrone. De plus, nous pourrions envisager des modifications en *ortho* et *méta* du phényl.

Le deuxième objectif de mes travaux de Thèse était d'améliorer la biodisponibilité et le ciblage des nitrones en les insérant sur des structures spécifiques. Nous avons choisi dans un premier temps de poursuivre les recherches menées au laboratoire sur un modèle de transporteur monomoléculaire amphiphile à fixation latérale. Ce concept de vectorisation qui repose sur le greffage d'un antioxydant sur la chaîne latérale d'un acide aminé lui-même relié à une partie hydrophile et une chaîne hydrophobe, a démontré par le passé qu'il était tout à fait généralisable à différents antioxydants ou agents thérapeutiques. Nous avons donc trouvé opportun de développer de nouveaux transporteurs amphiphiles sur lesquels plusieurs antioxydants pourraient être greffés. Ainsi, deux lysines ont été insérées au cœur de cette structure, soit avec des protections similaires sur leurs chaînes latérales, soit protégées orthogonalement. Deux nouveaux composés amphiphiles ont alors été synthétisés, l'un comportant deux motifs PBN et le second combinant dans sa structure une PBN et un Trolox,

analogue synthétique de la vitamine E. Les études des propriétés physico-chimiques ont permis de confirmer que greffer un ou plusieurs antioxydant(s) sur un transporteur amphiphile améliore à la fois la solubilité dans l'eau et la lipophilie globale de l'antioxydant. Les premiers résultats biologiques ont montré de meilleures activités antioxydantes pour le composé FADiPBN portant deux motifs nitrones que pour son analogue comportant une seule PBN. L'activité du conjugué mixte FATxPBN contenant une PBN et un Trolox est actuellement en cours d'étude.



La conception d'une telle structure amphiphile ouvre la possibilité à diverses stratégies. Par exemple, il serait envisageable d'incorporer plusieurs lysines consécutives afin d'étudier la multivalence de la PBN. De plus, la structure divalente mise au point dans ce travail, pourrait également être fonctionnalisée par d'autres types d'antioxydants. Enfin, en combinant les résultats obtenus dans les chapitres II et III, nous pourrions envisager la synthèse d'un nouveau composé dans lequel la PBN serait substituée par un groupement amide en β de la fonction nitrone et reliée à un transporteur amphiphile par une liaison éther située en *para* du phényl de la PBN. Ainsi, nous pourrions espérer améliorer les propriétés intrinsèques de la PBN tout en lui assurant une meilleure biodisponibilité et un ciblage plus spécifique.

APPENDICES

Partition coefficient (log k'_W)

The capacity of an active compound to reach its intended target in the body within any degradation is of particular interest to determine its pharmacologic efficacy. However, during its transport through the body, the active compound crosses hydrophilic and hydrophobic media such as for example blood serum or membranes. Following this principle, Hansch has developed a technique to determine how a compound divides between two mixtures and defined the partition coefficient, marked as **P**.[1-3] This is the ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium. The first solvent is water and the second is the hydrophobic 1-octanol, used to mimic the lipid membrane. The partition coefficient is defined as the difference of solubility of the compound in these two phases, a useful parameter in estimating the distribution of drugs within the body, which can be determine following this equation:

$$\mathbf{P} = \frac{[\text{compound}]\text{oct}}{[compound]aq\ (1-\alpha)}$$

α is the degree of dissociation of compound in water and **P** is experimentally determined by decantation, measuring the compound concentration in variable volumes of octanol and water. Collander has demonstrated that organic compound motions are proportional to partition-coefficient logarithm[4] and a new equation was then established by Hansch[5] as:

$$\text{Log } 1/C = -k\ (\log P)^2 + k'\ (\log P) + k''$$

in which C is the molar concentration for a standard biological response and k, k' and k'' are constants determined using the least square method. This relation allows to conclude that hydrophobic drugs with high octanol/water partition coefficients are preferentially distributed to hydrophobic compartments such as lipid bilayers. Conversely, hydrophilic drugs with octanol/water partition coefficients near to zero are preferentially found in aqueous compartments such as blood serum.

Determination of partition coefficient (log k'_w). High-performance liquid chromatography is a faster method of log **P** determination. This faster and easy technique needs relatively low quantities of product and can be applied to a wide range of organic compounds. The principle is based on the compound capacity to interact with the alkyl chains of silica gel, and on the mobile phase polarity. The retention time, which is specific to each compound at different mobile phase polarity, is strongly affected by these two parameters. After determining the retention time, the *k'* constant can be obtained using the following relation: $k' = (t_R - t_0)/t_0$ in which t_R and t_0 represent the retention time of the eluted compound and of the mobile phase, respectively. When plotting the log *k'* with the percentage of methanol in the mobile phase, a linear regression curve is obtained and intersection with the ordinate axe gives the theoretical value of log *k'* eluted with 100% of H_2O, denoted as log k'_w. An example is shown in Figure A.1 for the log k'_W determination of PBN. The retention times of the compounds described in the first part of chapter III are listed in Table A.1.

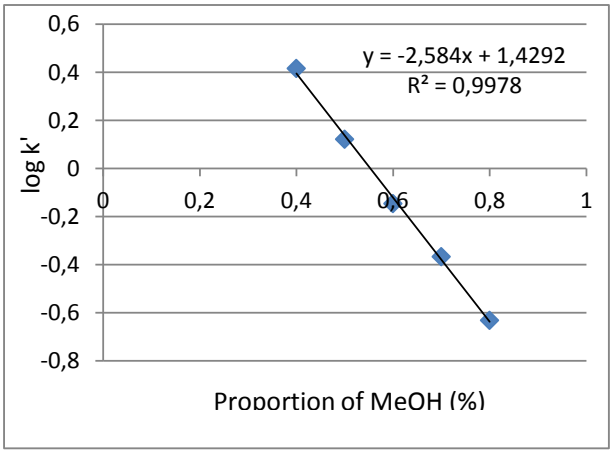


Figure A.1. Determination of log k'_W of PBN.

Compounds	Num	t_R					log k'					R^2	log k'_W
Eluent		MeOH/H_2O					MeOH/H_2O						
		90/10	80/20	75/25	70/30	60/40	90/10	80/20	75/25	70/30	60/40		
PBN		-	4.40	-	4.94	6.20	-	-0.63	-	-0.37	-0.14	0.9978 [a]	1.43 [a]
Trolox		4.21[a]	4.35[b]	-	4.84 [b]	-	-0.79 [b]	-0.76 [b]	-	-0.52[b]	-	0.9981 [b]	1.34 [b]
FAPBN		3.81	4.49	5.39	7.84	25.03	-1.02	-0.50	-0.18	0.10	0.77	0.9964	4.30
FADiPBN	7	3.94	4.96	-	10.17	47.82	-0.87	-0.34	-	0.28	1.09	0.9910	4.92
FATxPBN	10	4.03	6.22	9.95	21.28	-	-0.92	-0.12	0.30	0.69	-	0.9998	6.37
Rf-Lys(PBN)-OH	13	3.86	5.08	6.46	10.88	44.76	-0.95	-0.31	-0.02	0.33	1.05	0.9981	5.08
Rf-Lys(PBN)-OtBu	12	4.21	6.91	11.03	24.09	-	-0.67	0.01	0.37	0.77	-	0.9987	5.78
Rf-[Lys(PBN)]$_2$-OtBu	17	4.18	6.78	11.54	23.90	-	-0.69	0	0.40	0.75	-	0.9991	5.81
Boc-Lys(PBN)-Rf	14	4.15	6.46	10.10	18.62	-	-0.71	-0.05	0.32	0.64	-	0.9997	5.38
Boc-[Lys(PBN)]$_2$-Rf	18	4.25	7.21	12.40	26.33	-	-0.65	0.05	0.44	0.82	-	0.9992	5.94

Table A.1. Retention time and log k'_w values of nitrone derivatives. [a] Results obtained with five points, values in MeOH/H_2O 50/50 and 40/60 are not represented in this table; [b] Values from the literature.[6]

Cyclic Voltammetry (CV)

Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions because it offers a rapid and easy characterization of redox electroactive species. The principle consists to measure the variation of faradic current against the voltage variation applied to the redox system. The resulting potential produces an excitation signal which is measured between two voltage values, starting from the initial potential to the extrema ended potential, through a linear potential variation and at fixed rate. When the voltage reaches the extrema potential, also called switching potential, the reverse scan occurs and the voltage is swept back to the initial potential. This cycle can be repeated and the scan rate can be varied.

Determination of electrochemical potential. To obtain this variation, the voltammetric system might be constituted of the studied electrolyte, the solvent, an electrolyte to provide ions and ensure sufficient conductivity as well as a specific three-electrode setup composed of:

- A working electrode constituted of glassy carbon, on which the reaction of interest is occurring; the working electrode's potential is varied linearly with time,
- A reference electrode constituted of a silver wire and with constant concentration of AgCl solution, which maintains a constant and well-known electrode potential,
- An auxiliary electrode also called the counter electrode, which conducts electricity from the signal source to the working electrode.

A CV system consists of an electrolysis cell, a potentiostat, a current-to-voltage converter, and a data acquisition system. The current-to-voltage converter measures the current, and the data acquisition system produces the resulting voltammogram.[7, 8]

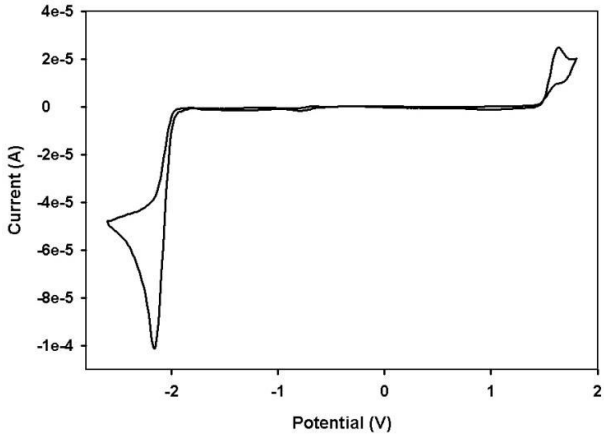
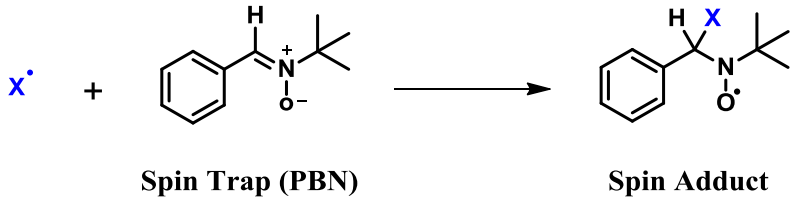


Figure A.2. Cyclic voltammetry of PBN in acetonitrile containing 50 mM of TBAP at 0.1mV/s.

Electron Paramagnetic Resonance (EPR)

Spin-trapping by EPR spectroscopy is a popular method for the detection and identification of short-lived free radicals in biological and chemical systems. EPR spectroscopy detects paramagnetic species such as unpaired electron of free radicals but when the half-life of radicals is too short, they generally cannot be detected by EPR. This is particularly true when stationary concentration of radicals studied is lower than the limit detection of EPR spectrometers used (about 10^{-8} $mol.L^{-1}$). The spin-trapping method consists in using a diamagnetic compound able to covalently react with fleeting free radicals and form a more stable paramagnetic spin adduct, whose half-life is significantly longer than that of the parent radical, and which can be observed and characterized by EPR spectroscopy. In the case of nitrones, the spin-adduct observed is a relatively stable radical species, called nitroxide.



Spin Trap (PBN) **Spin Adduct**

Figure A.3. Schematic representation of spin-trapping reaction using PBN.

Efficacy of the spin-trapping reaction depends to the following conditions:

- the spin-trap must be chemically stable in the experimental conditions,
- the rate of the free radical $R^{\bullet}$ addition onto the spin-trap must be high enough,
- the spin-adduct formed must be sufficiently persistent to be observed,
- the spin-trap must specifically react with the free radical R•.

Interpretation of EPR spectrum. EPR spectrometers measure the absorption of electromagnetic radiation. A phase-sensitive detector is used in EPR spectrometers which converts the normal absorption signal to its first derivative which is represented in the spectrum obtained. In the EPR spectrum, the magnetic field is on the x-axis in Gauss (G) unit

(1 Tesla = 10000 Gauss) whereas the derivative of the imaginary part of the molecular magnetic is on the y-axis, in arbitrary unit. EPR spectra are often very complicated and a computer program is used to analyze the spectra. The experimental spectrum is simulated using specific software such as for example the WINSIM program, available as free software from Public Electron Paramagnetic Resonance Software Tools (http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/). Through the simulated spectrum, important parameters can be determined such as the hyperfine coupling constant which is analogous to spin-spin coupling in NMR spectroscopy. The multiplicities of lines in the EPR spectrum of a spin adduct result from hyperfine interactions between the unpaired electron and cores of diamagnetic moment. Intensities of the spectral lines follow Pascal's triangle for I = 1/2 nuclei, similar to J-coupling in NMR. For example, when the linear nitrone PBN traps methyl radical, we obtain a six lines spectrum as represented in Figure A.4. Due to the hyperfin coupling with the nitrogen element (spin $I_N = 1$) the spectrum includes three lines which are then spitted because of hyperfine interactions with the hydrogen (spin $I_H = ½$) in ß position.

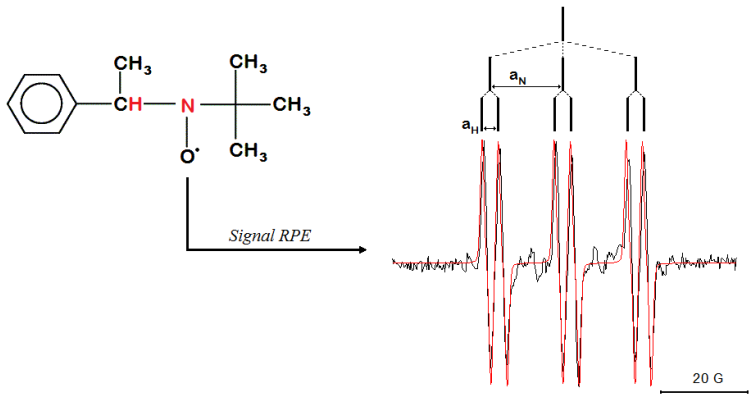


Figure A.4. PBN-CH_3 adduct spectrum (in black) obtained after trapping methyl radical in water, simulated spectrum (in red).

Critical Micelle Concentration (CMC)

Amphiphilic compounds are surfactants and one of their main characteristic is to self-assemble in aqueous environments to form small aggregates also called micelles. When placed in an immiscible biphasic system composed of aqueous and organic solvents, the amphiphilic compound will initially partition into the interface, between the two phases, reducing the system free energy. Subsequently, when the surface coverage by the surfactants increases and the surface free energy has decreased, the surfactants start aggregating into micelles. Consequently, the system free energy decreases again by decreasing the contact area of hydrophobic parts of the surfactant with water. A schematic diagram of the different steps which characterize micelles formation is represented in Figure A.5. The critical micelle concentration (CMC) is defined as the concentration of surfactants above which micelles are spontaneously formed (B) and all additional surfactants added to the system go to micelles. Before reaching the CMC, the surface tension changes strongly with the concentration of the surfactant (A). After reaching the CMC, the surface tension remains relatively constant or changes with a lower slope (C).

Self-association depends on the molecular structure of the drug, concentration, and physicochemical conditions such as temperature, pressure, pH, ionic strength, and on the presence and concentration of other surface active substances and electrolytes. Various shapes of aggregates are possible such as spherical, ellipsoidal or cylindrical.

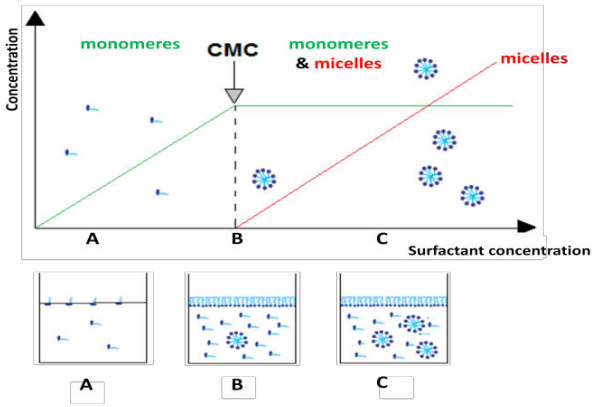


Figure A.5. Evolution of surfactant concentration around the critical micelle concentration.

Determination of critical micelle concentration. A large number of methods have been applied to determine the critical micelle concentration of surface-active agents. Most of the physico-chemical properties changes can be used to determine the CMC, provided that the measurement can be carried out accurately. For example, tension-surface, UV-Vis spectroscopy, luminescence spectroscopy, and electrical conductivity can be used. In this manuscript, CMC of fluorinated amphiphilic compounds was determined using ^{19}F NMR spectroscopy. This technique is easily achieved and the NMR spectra obtained are relatively simple. Experiments can be directly carried out in water with only 10% of D_2O, avoiding possible CMC shift in 100% of D_2O. The chemical shifts obtained in isotropic or anisotropic conditions and at different concentration are directly correlated to the nuclear spin interactions between molecules. At high concentration and under anisotropic conditions, micelles are formed and a broad and shielded NMR signal is observed. Under isotropic conditions there are only monomers in the solution, resulting in narrow and deshielded peaks, as represented in Figure A.6.

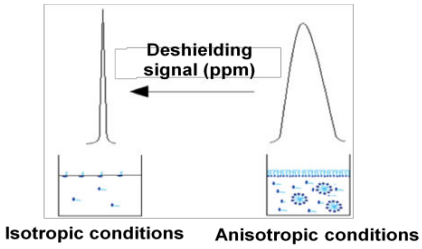


Figure A.6. Chemical shift variation from anisotropic conditions to isotropic conditions.

A concentrated solution of the fluorinated amphiphilic compound is firstly prepared in H_2O/D_2O (9:1) containing sodium trifluoroacetate salt (1 mg/1 mL) as interne standard and successive dilutions are then prepared. The trifluoroacetate peak is used to calibrate the spectrum. To determine the CMC value of our perfluorinated amphiphilic compounds, the last fluorinated carbon (CF_3) of the hydrophobic chain was chosen to measure the chemical shift. This typical peak is isolated compare to the other fluorinated signals and is usually observed around -82 ppm. After plotting the inverse concentration with the chemical shift values, we obtained two linear curves and their intersection gives the CMC value, as shown in Figure A.**7** for FAPBN.

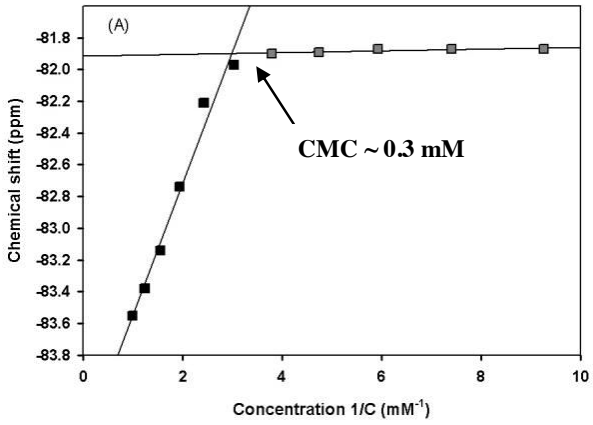


Figure A.7. CMC measurement of FAPBN.

Dynamic Light Scattering (DLS)

Dynamic light scattering is a well-known technique used to determine the size distribution profile of small particles in suspension or polymers in solution.[9] Small molecules in solution are undergoing random motion called Brownian motion and when a light hits small molecule, the light scatter in all directions. Dynamic Light Scattering works by measuring the intensity of light scattered by the molecules in the sample as a function of time. Since all molecules in solution diffuse with Brownian motion in relation to the detector, there will be interference which causes a change in light intensity. This leads to time-dependent fluctuations in the intensity of the scattered light. In DLS, the time scale of light intensity fluctuations are measured by a fast photon counter and can provide information regarding to the average size, size distribution, and polydispersity of molecules and particles in solution. Fluctuations are directly related to the rate of the diffusion of the molecule through the solvent and the translational diffusion coefficient D of the particles can be determined. With this information, the particle size can be determined by calculation of the hydrodynamic diffusion coefficient D_H following the Stock-Einstein relation:

$$D = k_B T / 3\pi\eta D_H$$

in which k_B : Boltzmann constant; T : absolute temperature; η : viscosity of the solvent; D : transitional diffusion coefficient.

Determination of Dynamic light scattering.

In practice, a first solution is prepared at concentration more than ten times the CMC, diluted solutions are then prepared and each sample is analyzed using a Nanosizer ZS apparatus. Once the autocorrelation data is generated and after different mathematical approaches employed by the software, we obtain information such as, for example, volume distribution or intensity distribution.

References

[1] C. Hansch , T. Fujita A method for the correlation of biological activity and chemical structure, J. Am. Chem. Soc., 86 (1964) 1616-1626.

[2] C. Hansch , T. Fujita , J. Isawa A new substituent constant, π, derived from partition coefficients, J. Am. Chem. Soc., 86 (1964) 5175-5180.

[3] C. Hansch , P.P. Maloney , T. Fujita , R. Muir Correlation of biological activity of phenoxyacetic acids with Hammett substituent constants and partition coefficients, Nature, 194 (1962) 178-180.

[4] R. Collander, The permeability of Nitella cells to nonelectrolytes, Physiologia Plantarum, 7 (1954) 420-445.

[5] C. Hansch , A.R. Steward , S.M. Anderson , D. Bentley Parabolic dependence of drug action upon lipophilic character as revealed by a study of hypnotics, J. Med. Chem., 11 (1968).

[6] S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M.A. Pappolla, J. Boeker, R. Hardeland, B. Pucci, Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents, J. Med. Chem., 49 (2006) 2812-2820.

[7] D. Skoog, F. Holler, V. Crouch, Principles of Instrumental Analysis (2007).

[8] P.T. Kissinger, W.R. Heineman, Cyclic Voltammetry, J. Chem. Educ., 60 (1983) 702.

[9] B.J. Berne, R. Pecora, Dynamic Light Scattering, Courier Dover Publications (2000).